COMPARISON OF MULTIPLE DRUG AND METABOLITE LEVELS RECOVERED FROM SKELETONIZED REMAINS FOLLOWING STANDARD PASSIVE EXTRACTION, MICROWAVE-ASSISTED EXTRACTION AND ULTRASONIC SOLVENT EXTRACTION AND GC-MS OR UPLC-DAD

by

CAROLINE BETIT

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Chemical Sciences

The School of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE

Laurentian Université/Université Laurentienne
School of Graduate Studies/École des études supérieures

Title of Thesis Titre de la thèse	COMPARISON OF MULTIPLE DRUG AND METABOLITE LEVELS RECOVERED FROM SKELETONIZED REMAINS FOLLOWING STANDARD PASSIVE EXTRACTION, MICROWAVE-ASSISTED EXTRACTION AND ULTRASONIC SOLVENT EXTRACTION AND GC-MS OR UPLC-DAD
Name of Candidate Nom du candidat	Betit, Caroline
Degree Diplôme	Master of Science

Department/Program Département/Programme	Chemical Sciences	Date of Defence Date de la soutenance	January 31, 2014

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. James Watterson
(Supervisor/Directeur de thèse)

Dr. Nelson Belzile
(Committee member/Membre du comité)

Dr. Joy Gray-Munro
(Committee member/Membre du comité)

Dr. Jeffery Hackett
(External Examiner/Examinateur externe)

Approved for the School of Graduate Studies
Approuvé pour l'École des études supérieures
Dr. David Lesbarrères
M. David Lesbarrères
Director, School of Graduate Studies
Directeur, École des études supérieures

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

I, **Caroline Betit**, hereby grant to Laurentian University and/or its agents the non-exclusive license to archive and make accessible my thesis, dissertation, or project report in whole or in part in all forms of media, now or for the duration of my copyright ownership. I retain all other ownership rights to the copyright of the thesis, dissertation or project report. I also reserve the right to use in future works (such as articles or books) all or part of this thesis, dissertation, or project report. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that this copy is being made available in this form by the authority of the copyright owner solely for the purpose of private study and research and may not be copied or reproduced except as permitted by the copyright laws without written authority from the copyright owner.

Abstract

Optimization and characterization of microwave-assisted extraction (MAE) and ultrasonic-solvent extraction (USE) of drugs from bone was developed and compared to standard passive extraction. Extraction solvent, sample mass, extraction time, solvent volume, microwave power and presence of dissolved gasses in bath-water and extraction solvent were investigated. Solvent extracts were assayed by GC-MS (EI-SIM) or ULPC-DAD.

Higher yields were obtained with methanol while sample mass had no effect on analyte recovery. Maximum yield was achieved within 30, 15 and 45 min of passive extraction, MAE and USE, respectively. Solvent volume did not influence analyte recovery for MAE, but larger and smaller solvent volumes obtained higher yields for passive extraction and USE, respectively. Higher microwave power resulted in greater recovery and degassed bath-water and extracting solvent resulted in significantly higher recovery. Higher recovery was achieved with passive extraction, MAE and USE are more time efficient, and more cost efficient and environmentally friendly, respectively.

Keywords: Forensic toxicology, bone, microwave-assisted-extraction, ultrasonic-solvent extraction, drug, GC-MS, UPLC-DAD.

Acknowledgments

First of all, I would like to thank my supervisor, Dr. James Watterson. Thank you for all your guidance and immense patience, understanding and motivation. Your passion for research and teaching was reflected in the amount of time you put into the multiple discussions we had, the advice and knowledge you shared and the continuous help and support you provided throughout the different steps of my thesis. Thank you for providing me with the opportunity to further my education and for encouraging me to step out of my comfort zone. For this reason I have accomplished more than I ever thought possible. You have my utmost respect and my sincere gratitude. I could not have asked for a better supervisor! I hope to one day be able to offer the same inspiration and support to someone else, like you have done for me throughout my education.

I would also like to thank Dr. Nelson Belzile and Dr. Joy Gray-Munro for your participation in my thesis committee. Your advice, guidance and feedback during the annual committee meetings was greatly appreciated, thank you.

Thank you to Dr. John Wyman from Ohio and to Chris Tindall from Costco Wholesale for supplying the pig carcase and the control bone, without which my thesis would not have been possible. I would also like to acknowledge the Nature Sciences and Engineering Research Council of Canada and the Midwest Forensics Resource Center for financial support.

In addition, I would like to thank my friends Nathalie Desrosiers for helping me with the writing process and for your enthusiasm and motivation which made the harder days a little easier, Brittany Findlay-Mitchell for your patience and understanding during the many nights I came

back to the apartment needing to vent and unwind and Mike Pretz for the distractions when I needed a change of scenery – it made it that much easier to come back to work after a little diversion. Last but not least, I want to acknowledge my family, Martine Durand, Gilles Betit and Lyanne Betit. Thank you for your continuous support and motivation through these years. Your kind and encouraging words, along with your faith in me, have led me to become who I am today. I would not have made it here without you; thank you so much!

Table of Contents

List of Tables

List of Figures

Glossary

% CV:	Percent coefficient of variation
6AM:	6-monoacethylmorphine
ACN:	Acetonitrile
ADHD:	Attention deficit hyperactivity disorder
Adsorption:	Accumulation of gas or molecules on the surface of a solid.
AMI:	Amitriptyline
ANOVA:	Analysis of Variation
Anticholinergic effect:	Symptoms resulting from agents that inhibition of the action of acetylcholine
BTE:	Bone Tissue Extract
BZE:	Benzolecgonine
Canaliculi:	Mode of communication of osteocytes with adjacent osteocytes and blood supply allowing diffusion of oxygen, nutrients and waste into bone matrix
CCE:	Cocaethylene
CI:	Chemical Ionization
CIT:	Citalopram
CNS:	Central Nervous System
Conduction:	Transfer of heat between substances that are in direct contact.
Convection:	Transfer of heat through a gas or liquid.
DCIT:	Desmethylcitalopram
DDCIT:	Didesmethylcitalopram
Demineralization:	Process of removing inorganic material

Desorption:	Change of an adsorbed state on a surface to a gaseous or liquid state
Dg H_2O:	Degassed water
Dg MeOH :	Degassed methanol
DMI:	Desipramine
Dysrhythmia:	Abnormality in physiological rhythm in activity of brain or heart.
EA:	Ethyl Acetate
EI:	Electron Impact
Enuresis:	Involuntary urination at night
Fibromyalgia:	Chronic disorder characterized by musculoskeletal pain and fatigue
FID:	Flame Ionization Detector
First-Pass Effect:	Phenomenon where drugs taken orally enter the circulatory system and undergo metabolism before being distributed to the rest of the body.
G H_2O:	Gassed water
G MeOH:	Gassed methanol
Gavage:	Administration of food or drugs by force through a tube leading down the throat to the stomach
GC-MS:	Gas Chromatography-Mass Spectrometry
Half-Life:	Time required for half of the drug to be metabolized.
Homogenization:	Act of making something uniform in composition.
HPLC:	High-Performance Liquid Chromatography
Hydrolysis:	Chemical breakdown of a compound by reaction with water
IC:	Intracardiac
IM:	Intramuscular

IP:	Intraperitoneal
ISTD:	Internal Standard
IV:	Intravenous
Lacuna:	Small space where osteocytes are located with bone.
LC-MS:	Liquid Chromatography-Mass Spectrometry
Lipophilicity:	Capability to dissolve in lipids.
LLE:	Liquid-Liquid Extraction
LOQ:	Limit of Quantitation; Lowest concentration following validation criteria of precision (% CV < 20) and accuracy.
MAE:	Microwave-Assisted Extraction
MAOI:	Monoamine Oxidase Inhinbitor
Matrix Effects	Response due to endogenous compounds found in the matrix.
MeOH:	Methanol
m/z:	Mass to charge ratio
NLBP:	Nucleation Limited Boiling Point
Nocturia:	Excessive urination at night
NPD:	Nitrogen-Phosphorous Detector
NTRIP:	Nortriptyline
OCD:	Obsessive-Compulsive Disorder
OCPs:	Organochlorine Pesticides
Osteon:	AKA Harversian system, made of concentric rings of calcified extracellular matrix, lamellae, surrounding a Haversian canal.
PAHs:	Polycyclic Aromatic Hydrocarbons
Pharmacodynamics:	Branch of pharmacology concerned with the effect of the drugs and

	their mechanism of action on the body.
Pharmacokinetics:	Branch of pharmacology concerned with the movement of drugs within the body and its fate.
PB:	Pentobarbital
PBS6:	Phosphate Buffered Saline at pH 6
PCBs:	Polychlorinated Biphenyls
RR:	Response Ratio
RR/m:	Mass-normalized Response Ratio
SB:	Secobarbital
SIM:	Selected Ion Monitoring
SPE :	Solid-Phase Extraction
SSRI:	Selective Serotonin Reuptake Inhibitor
Silylation:	Addition of a substituted silyl group
Tachycardia:	Abnormally rapid heart rate.
TCA:	Tricyclic Antidepressant
Tensile strength:	Maximum tension a material withstand without tearing.
Thermolabile:	Substance destroyed or deactivated by heat.
TMPAH:	Trimethylphenyl Ammonium Hydroxide
UPLC-DAD :	Ultra-Performance Liquid Chromatography with Diode Array Detector
USE :	Ultrasound Solvent Extraction
UV:	Ultra-Violet

Volume of Distribution (Vd): Apparent volume in which a drug distributes in the body.

Chapter 1

1 Introduction

1.1 General Introduction to Forensic Toxicology

Forensic toxicology is a branch of forensic science which applies principles of different disciplines, such as pharmacology, analytical chemistry and clinical chemistry, to assist legal and medical investigations of drug use, poisoning and death. Toxicological analysis of samples may determine the role of drugs in medico-legal death investigations, in cases of unnatural and suspicious deaths.

1.2 Post-Mortem Toxicology Samples

Conventional specimens collected at autopsy for toxicological analysis include bodily fluids such as blood (central [ex: heart] and peripheral [ex: femoral]), urine, vitreous humor, stomach contents, bile, and organs such as liver, kidneys, spleen, lungs and brain [1,2]. In situations involving animal scavenging, advanced decomposition or skeletonization, conventional tissues may be unavailable. Hair, nails, teeth and bone may be the only biological samples remaining that can potentially be used for toxicological analysis [1,2,3].

After collection of specimens, samples must undergo a series of steps prior to analysis, where sample preparation is often the longest and most important step of a drug testing procedure. The goals of sample preparation are to concentrate the targeted analytes, clean up the sample by

reducing interference from endogenous compounds in the matrix and/or separate the analytes from the biological matrix and transfer them to a solvent amenable for the analysis.

Some of the most commonly used procedures in sample preparation include protein and lipid precipitation, solid-phase extraction (SPE) and liquid-liquid extraction (LLE). Each technique takes advantage of the physiochemical properties of the analyte(s), such as lipophilicity and pKa, to achieve solubility in different solvents in order to remove contaminating compounds and separate the targeted analytes.

Screening is often achieved by a combination of spectrometry, simple colour test, chromatography or immunoassay. Spectrometry can include spectrophotometry; chromatography uses the substance volatility and solubility for the partitioning between the mobile and stationary phases of gas and liquid chromatography, respectively. Immunoassay involves the competition between labeled drug and the drug or drug-class in the sample for the sites on selective binding antibodies [4]. Once the screening technique indicates the presence of potential analytes, confirmation must be established. Two of the most common confirmation techniques are GC-MS and LC/MS/MS.

1.3 Bone Anatomy

The human skeleton is a metabolically active organ that is composed of 206 bones and is a highly specialized form of connective tissue whose main goal is maintaining internal support and protecting organs. As a secondary function, bone is also a line of defence against acidosis [2,5]. Combined with bone marrow, skeletal tissue makes up approximately 14 % of the average

human body mass; a potentially large surface area for adsorption of toxins, metals and drugs [2,5,6]. The most abundant cell type in bone are osteocytes: these are surrounded by a mineralized extracellular matrix, composed mainly of calcium phosphate ($Ca_3(PO_4)_2$) combined with calcium hydroxide ($Ca(OH)_2$) to form crystals of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). On the other hand, osteoblasts, osteoclasts and bone lining cells are found on the surface of the bone [1,7]. Osteoblasts produce the bone matrix, rendering the tissue strong and rigid, with some degree of elasticity. Once surrounded by impermeable bone matrix, osteoblasts mature into osteocytes and become responsible for the maintenance of bone tissue and calcium homeostasis, through synthesis and resorption of the matrix [6]. Osteoscytes are located in small spaces called lacunae and communicate with adjacent osteocytes and blood supply, located at the interior and exterior bone surfaces, by canaliculi. This allows diffusion of oxygen, nutrients and waste, and deposition of drugs and metabolites into the bone matrix [8].

Bones are found in 4 different shapes in the human body: long, short, flat and irregular, and 2 different forms: cortical, also known as compact or dense bone, and cancellous, also called trabecular or spongy bone [1,6]. The difference between the 2 forms is both structural and functional. Compact bone has very little porosity (5-30 %), accounts for approximately 80 % of the total bone mass and has protective and mechanical functions. Compact bone is formed of substructures called osteons, also known as Haversian systems, which are organized in concentric rings of calcified extracellular matrix called lamellae. Each lamella surrounds a Haversian canal, where nerves and blood vessels run longitudinally through the bone (figure 1). These nerves and blood vessels branch off the Haversian canal into smaller channels, called canaliculi, that penetrate the compact bone in every direction and interconnect lacunae, small spaces where osteocytes reside [1,6,9].

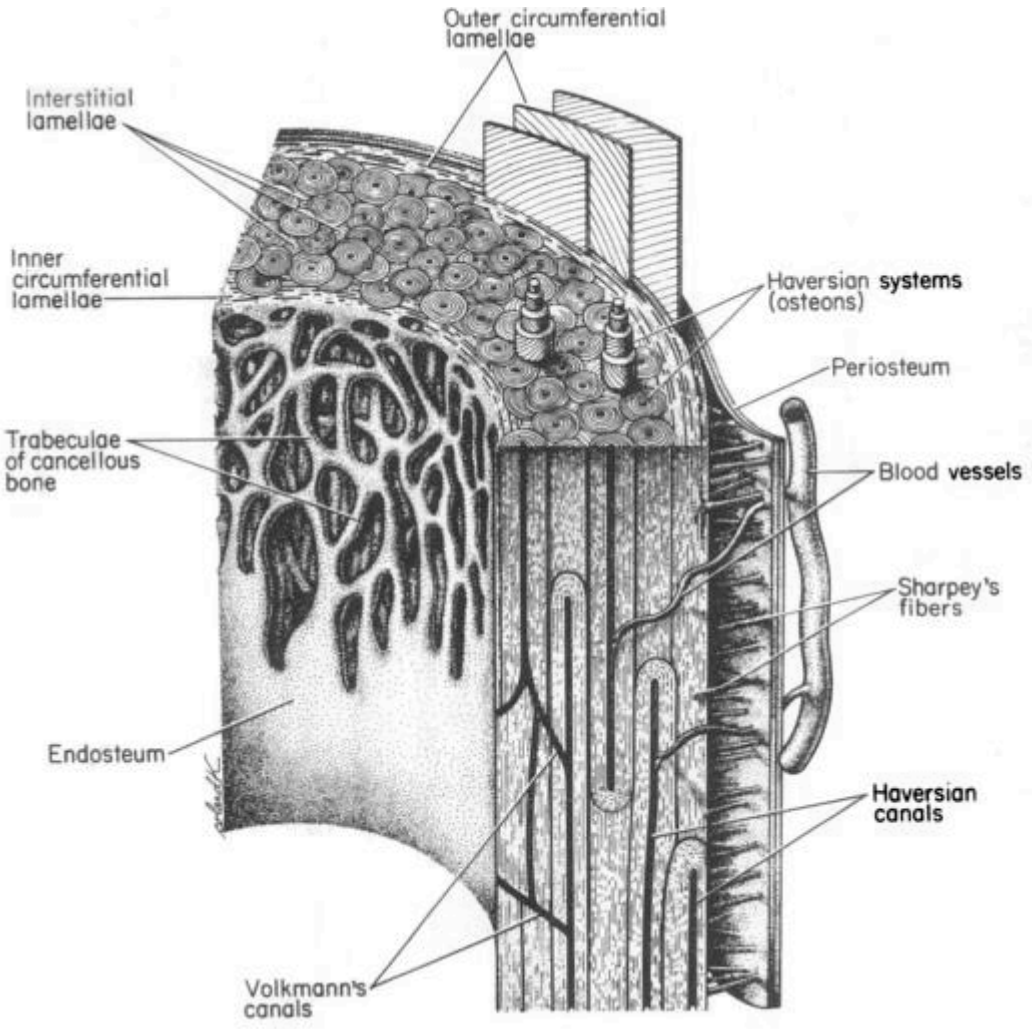


Figure 1: Sketch of typical long bone illustrating important features of cortical and cancellous bone (After Martin, 2007 [9])

Cancellous bone is formed of a network of trabeculae, which consists of lamellae arranged in an irregular pattern, rather than in osteons. This physical attribute makes the bone lighter and more porous (30-90 %) and creates a greater surface area for the exchange of waste and nutrients between osteocytes and blood vessels. Unlike compact bone, where extracellular fluid fills the space in the lacunae and canaliculi, bone marrow fills the space in between the bony rods, serving a metabolic function [1,6,8].

Bone marrow is also found in the medullary cavity and includes red and yellow marrow. Red bone marrow consist of connective tissue rich in vascular supply and contains lots of hematopoietic (blood stem cells) cells and white blood cells, in particular macrophages. Although yellow bone marrow consists of connective tissue and blood vessels as well, it also contains inert adipose cells (fat) [2]. Due to its high vascularity and lipid matrix, bone marrow can serve as a drug repository. Winek [10] has demonstrated a correlation between drug levels in plasma and bone marrow. For example, an increase of desipramine (DMI) levels in bone marrow was observed as the plasma drug levels also increased, showing that bone marrow could be used as an alternative tissue for toxicological analysis when blood is unavailable [10].

1.4 Drugs in Bone

Deposition of drugs in bone is not yet fully understood. Factors such as exact drug location and drug time course in bone are still poorly characterized. One can hypothesize that drugs enter bone through the circulation system, along with the nutrients and oxygen, transferring from the blood vessels into the canaliculi to reach the osteocytes. After death, bone would act as a weakly porous box trapping them inside. The drugs can also accumulate in the bone marrow due to its high vascularity and fatty matrix. Postmortem, bone marrow would eventually dehydrate during skeletonization, where the drugs would reside encased in different parts of bones. Finally, during decomposition and liquefaction of organs, drugs and metabolites primarily contained in soft organs would eventually be liberated and deposited onto the surface of the bones. Results of different studies looking at the distribution of drugs within a bone and between different bones showed varying levels of drug recovery [11,12,13,14].

Multiple factors can influence drug and metabolite distribution in bone and bone marrow, such as the time since last exposure, frequency of drug intake (acute versus chronic), bone type, and drugs' and metabolites' physiochemical characteristics. Some compounds may not accumulate or may not be transferred from blood to bone as easily as others due to a short half-life, polarity (especially metabolites) and protein-binding [1].

Results from McGrath's project on the detection of drugs of forensic importance in post-mortem bone demonstrated that in general, drugs were more likely to be detected in blood than bone and that a high blood concentration did not always result in high concentration in bone, but that certain drugs (ex: nortramadol, cocaethylene and quinine) were detected in higher level in bone. Therefore no relationship between drug concentration in blood and the probability of detecting the compounds in skeletal tissue could be established [1]. Drug deposition in skeletal tissue can therefore be drug dependent and should be investigated for each drug and its metabolites.

1.5 Techniques for Drug Isolation from Bone

Sample preparation is usually the most important and also the most time consuming step of an analysis. It includes many different techniques to extract, dissolve, dilute, concentrate, and clean up samples in order to increase the sensitivity and selectivity of the analysis.

The distribution and accumulation of drugs and their metabolites in bone has yet to be completely understood. For this reason, their recovery and measurement in bone is challenging and poorly characterized. Furthermore, extraction of drugs from solid matrices such as skeletal tissue, as opposed to bodily fluids, is more complex due to the heterogeneous solid matrix. As a

result, extra steps are required in order to homogenize and then isolate the analytes from the solid matrix prior to an extraction step. Additionally, matrix effects are commonly observed with this tissue type due to its large level of background components.

Different isolation techniques to recover drugs and metabolites from skeletal tissue have been attempted. The first method found in literature was by Terazana and Takatori in 1982, where the cortical part of a humerus from a buried body, with an estimated post-mortem interval of 2-5 years, was used to perform acid digestion [15]. Acid digestion was accomplished with 1 g of ground bone added to 16 mL of 3 N HNO_3 for 18 h at room temperature to demineralise the bone. This technique has since been used in multiple reports with slight changes to the procedure. Raikos et *al.* detected opiates from a femur of a fatal poising case and compared levels with a piece that was later buried for a year. In this case, demineralization was carried out similarly but for a total of 24 h [16]. Guillot et *al.* also used acid incubation to perform bone hydrolysis to recover the analytes; in this case 0.1 M HCl was used on 50 mg of bone at 55 °C for 12 h [3].

Soxhlet extraction has also been used for the extraction of drugs and metabolites from skeletonised tissue. In 1985, Banko reported the extraction of amobarbital and glutethimide from multiple organs, including bone, from 10 bodies where poisoning was evident at autopsy. Reflux was performed for 1 h with 96 % ethanol at pH 3 [17]. Wohlenberg reported using soxhlet extraction for nortriptyline extraction from cancellous vertebra bones of a suicide victim; bones were cut into 1-2 cm pieces and extracted with 350 mL of methanol overnight (15 h) [18].

1.6 Standard Passive Extraction

Standard passive extraction has been the most widely applied method for the isolation of drugs and metabolites from skeletal tissue in the past decade. Many report the use of this technique to extract a wide variety of drugs [1,2,11,12,13,19,20,21,22]. McIntyre et *al.* were amongst the first to document the recovery of drugs from human mid-femoral bone from case work, which included therapeutic drug ingestions and presumptive drug overdose [2]. A total of 12 drugs were detected, including antidepressants, antipsychotics and benzodiazepine tranquilizers. Drugs and primary metabolites were extracted by soaking 10 g of sectioned bone rings in 25 mL of methanol at 50 °C for a period of 18 h [2]. Horak [19,20] and McGrath [21] have followed and reported similar techniques, where 1-2 g of bone and bone slivers were incubated in 2-4 mL of methanol or water at room temperature for 16-24 h [1,19,20,21]. Since then, Watterson et *al.* [11,12,13,14,22,47,93,96] have reported the optimization of passive solvent extraction technique to allow the recovery of multiple drugs and metabolites possessing different chemical properties. As little as 0.2-0.3 g of ground bone with 2 mL of methanol can be sufficient to detect drugs in skeletal tissues [11,12,13,22]. In general, extraction was performed at 50 °C for a total of 12-72 h. It is presumed that ground bone yields higher analyte recovery due to the higher surface area of the solid matrix exposed to the extraction solvent.

Isolation techniques used for skeletal tissue have been adapted from environmental toxicology for the extraction of pesticides from sediments and soils and detection of lead, mercury and arsenic in bone [17]. Over time, these methods have also been modified to accommodate other matrices such as hair and teeth [18,23,24]. Acid digestion, Soxhlet extraction and standard passive extraction all have long incubation periods (12-72 h), limiting the analytical throughput.

1.7 Microwave-Assisted Extraction (MAE)

1.7.1 History and Applications

During World War II, radars were developed, which initiated microwave technology growth [25]. Originally, microwaves were applied to the treatment of coal in order to remove organic sulfur, as well as frozen food tempering and pasta drying. In the mid- 1970s, microwave ovens started to be mass produced due to the improvements to the magnetron, making this instrument more affordable to the general population [26].

Once microwave ovens were commercialized, they were first used domestically for cooking and scientifically for drying samples [27]. Hesek and Wilson were the first to use microwaves for analytical purposes. Before the appearance of microwave ovens, samples such as wet cakes were dried on a hot plate or in ovens for 3-4 h. Currently, with the use of microwaves, sample drying can be accomplished within 15 min of irradiation. Hesek and Wilson were the first to determine that the time required to dry a sample was dependent on the size of the sample and the number of samples irradiated simultaneously [27].

Abu-Samra et *al.* [28] were the first to use microwave technology for digestion in order to determine trace metals from biological samples, as an alternative technique for wet ashing (wet digestion) [29]. Today, this technique has been expanded to environmental, biological, geological and metallic matrices, including fly ash and coal [30].

However, it was only in 1986 that the first publication appeared; this report by Gedye et *al.*, documented the use of microwave irradiation to carry out organic chemistry reactions, such as catalytic hydrogenation of alkenes [31]. This report led to further development in microwave

technology, which led to a large publication increase in the mid 1990s [26]. Since then, microwave technology has been used for sample drying, digestion, moisture measurement, sample clean-up, analyte desorption-adsorption, chromogenic reactions, speciation, nebulization of sample solution, hydrolysis, and extraction [30,32,33].

1.7.2 Microwave-Assisted Extraction

The first publication documenting the use of microwave irradiation for extraction purposes was in 1986 by Geyde et *al.* [31]. This group utilized microwaves for the extraction of lipids and pesticides from soils, foods and seeds [30,31,34]. The extraction of organic contaminants with a laboratory-grade microwave oven was first introduced by Lopez-Avila et *al*. in 1994 [34]. Since then, microwave-assisted extraction (MAE) has been used for the extraction of pesticides and herbicides (including organochlorine pesticides [OCPs], polychlorinated biphenyls [PCBs]), polycyclic aromatic hydrocarbons (PAHs), neutral and basic pollutants, and phenols from sediments, soil and atmospheric particles [30,35,36]. Microwave irradiation has also been applied to extract vitamins, essential oils, medicinal and pharmaceutical products from foods, aromatic herbs and plant materials [34,37,38,39].

1.7.3 Theory behind Microwave Ovens

Microwaves are non-ionizing electromagnetic waves, between the radio and infrared waves, with a frequency range from 0.3 to 300 GHz [26,37]. Commercial microwave ovens normally operate at a frequency of 2450 MHz (wavelength of 12.2 cm) to avoid interferences and produces 0.23 cal/mol (0.963 J/mol) of energy (quantum energy of approximately 0.0016 eV, below any

chemical bond energy) [26,40,41]. At this frequency the electric field alternates 4.9 x 10^9 s^{-1} and the polar molecules begin rotating, trying to align with the constantly changing electric field. The field changes before the molecules can complete their rotation, and vibration, also known as rotational oscillation, occurs where heat is generated through friction [26,37,40]. At frequencies greater than 2450 MHz, the molecules do not have time to start realigning since the electrical field changes too rapidly. At frequencies lower than 2450 MHz, the electrical field changes much more slowly and the molecules have time to align themselves. In both of these situations, no heat is generated [37].

1.7.4 Microwave Heating

Microwave heating, the transformation of electromagnetic energy in thermal energy, is generated from the interaction of the electrical field with polar compounds and solvents. Microwave heating can occur in two mechanisms: ionic conductance and dipolar rotation. Ionic conductance is the migration of ions under an electric field. Friction is generated due to the interaction of migrating ions with the solvent, consequently producing kinetic energy and converting it into thermal energy [26]. The migration direction of the ions changes as many times as the electric field changes polarity [26,37,39,40]. Dipole rotation is defined by the constant realignment of dipolar molecules in a continually altering electric field. As the molecules agitate and collide, heat is created [26,37,39,40]. The ability of molecules to align themselves with the field depends on the microwave frequency and the viscosity of the liquid [26].

The efficiency with which a material absorbs microwave energy and transforms it to heat depends on its dissipation factor (tan δ). The following equation defines tan δ:

$$\tan \delta = \frac{\varepsilon''}{\varepsilon'}$$

where ε' represents the dielectric constant (relative permittivity), which measures the capacity of a molecule to be polarized by an electric field, and therefore to store potential energy, and ε'' represents the dielectric loss, which measures the efficiency of the conversion of electromagnetic energy into heat in a varying electrical field. Only dielectric materials or solvents with permanent dipole moments, such as water and methanol, can absorb microwave energy and produce heat (table 1) [37]. Non-polar materials, such as hexane, are transparent to microwaves, and thus do not generate heat [39]. The more polar the solvent, the greater the dielectric constant and therefore, the more efficiently microwave irradiation will be absorbed; allowing the system to achieve higher temperatures [26]. For example, as seen in table 1, methanol has a lower dielectric constant than water, but a higher dielectric loss, resulting in a higher dissipation factor. Thus, methanol may not absorb microwaves as well as water, but it has a better ability to dissipate the microwave energy into heat [26,40]. A common solvent for the extraction of polar compounds is methanol or a mixture of methanol and water [40].

Based on these phenomena, there are three possible mechanisms of MAE [39,42]. First, the analyte may be extracted into a solvent (or a mixture of solvents) that strongly absorbs microwave energy. Secondly, the analyte may be extracted into solvents with both high and low dielectric losses. Third, the analyte may be extracted into a microwave transparent solvent from a sample matrix with a high dielectric loss.

Table 1: Chemical and physical properties, including dissipation factors, of solvents with different microwave irradiation absorbing abilities at 25 °C.

Solvents	Dielectric constant (Ɛ')	Dielectric loss (Ɛ'')	Dipole moment	NLBP (°C)*	References
Acetone	20.7	0.95	2.69	81	26,30,43,44
Acetonitrile	37.5	1.65	3.44	107	30,44
Ethanol	24.3	1.625	1.69	103	30,40,44
Methanol	32.7	15.296	1.70	84	30,40,43,44
Heptane	1.9	0.00019	<0.01	N/A**	40
Hexane	1.89	0.000019	< 0.1	80	30,43,45
2-Propanol	19.9	2.81	1.66	100	30,44
Ethyl acetate	6.02	0.3	1.78	95	26,43,44
Water	80	10	1.87	104	30,40,43,44

* Nucleation limited boiling point
**Not available

1.7.5 Superheating

A solvent irradiated in a closed-vessel system can reach a temperature higher than its boiling point due to the increased pressure and the lack of nucleation sites. This phenomenon is called superheating [40,44]. Baghurst and Mingos [44] referred to this temperature as the nucleation limited boiling point (NLBP) [44].

The boiling process depends on nucleation sites, cavities, pits and scratches on the vessel and in the solid sample matrix. The formation of bubbles during the boiling process rely on the vapour (embryo) trapped inside a crevice by the surrounding liquid. A bubble is created when the temperature of the solvent reaches the temperature corresponding to the pressure inside the embryo. The growth of the bubble is due to the evaporation of the superheated solvent around the bubble. Once the force holding the bubble in place is overcome, the bubble is released and boiling begins (figure 2) [44].

The number of active sites depends on the surface of the vessel and/or matrix and the solvent's ability to wet the surface. The larger the number of active sites, the lower the NLBP. Organic solvents have good wetting properties, trapping less vapour, forming a lower number of nucleation sites and leading to a higher NLBP. On other hand, water has a poor wetting ability and therefore a high number of embryo and a lower NLBP (see Table 1 for examples of NLBP) [44].

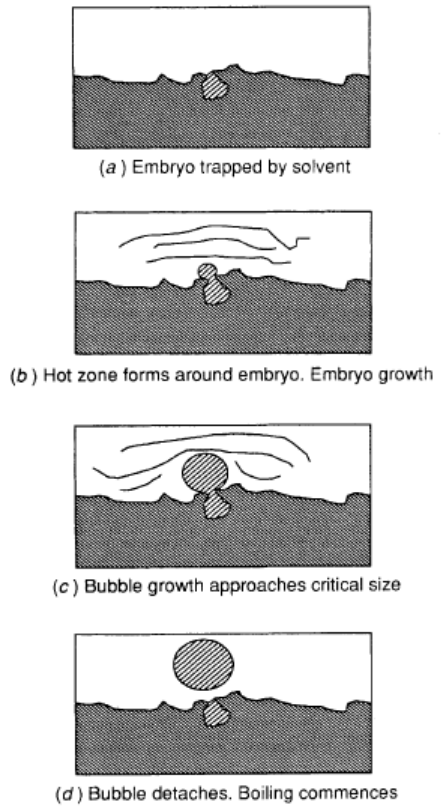


Figure 2: Increasing the nucleation liquid boiling point (Baghurst and Mingos, 1992) [44]

In conventional heating, a conduction/convection phenomenon occurs where thermal energy is supplied externally to the walls of the vessel (conduction) which, in turn, is transferred within the liquid (convection). A large part of this heat produced is lost to the environment [29,39]. During

microwave irradiation, the matrix or the solvent is directly heated (internal heating), with practically no heat lost (in closed-vessel system). This leads to shorter extraction times [37]. Therefore, in conventional heating, the superheated solvent layer is only in contact with the vessel, as opposed to microwave heating where the bulk solvent is superheated [29,39]. Having the bulk of the solvent reach NLBP rather than only the layer adjacent to the vessel is an important aspect in understanding the mechanism and efficiency of microwave heating [44]. Thus, the rapidity of MAE compared to standard passive extraction also depends on the conditions of the surface of the glass vessels and/or the sample matrix and the wetting properties of the extraction solvent [44].

In 1992, Baghurst and Mingos verified this theory by adding different concentrations of detergent to water samples [44]. Results show an enhancement in the wetting ability and a higher NLBP, even with the lowest detergent concentration, compared to water only. On the other hand, NLBP were reduced when water was added to ethanol samples and when the wetting properties of glass were reduced by silylation with trimethylsilyl chloride ($(CH_3)_3SiCl$) [44]. Different ways to increase the heating rate of a solvent include the addition of small amounts of organic solvent with a high dissipation factor to a non-polar solvent, where the heat transfer between the solvents is quick, or with ionic liquids by addition of salt to solvents [26].

1.7.6 Influential Parameters

1.7.6.1 Extracting Solvent

The solvent is chosen according to its selectivity for the analyte and its ability to extract and dissolve the analyte. The solvent must also be able to absorb microwave energy and should not

interact with the matrix [37,39,40]. Many organic solvents are flammable, and when heated, can combust or explode. For this reason, great caution must be taken when choosing an extraction solvent in order to prevent such occurrences.

1.7.6.2 Solvent Volume

The solvent volume depends on the type and the size of sample [39]. In comparison to conventional extraction techniques, where a high ratio of solvent volume to solid matrix is necessary, MAE, on average, requires about ten times less solvent [37,39]. The larger the solvent volume, the faster heat is generated, but Mandal [37] and Madej [39] reported lower recoveries with larger solvent volumes due to inadequate stirring.

1.7.6.3 Sample Mass

As the sample mass increases, so does the solvent volume required to ensure the sample is immersed throughout the entire irradiation time. Increasing the sample mass and solvent volume also increases the extraction time required to achieve maximum recovery [37,39].

1.7.6.4 Extraction Time

As mentioned previously, extraction time in MAE is much shorter than with conventional extraction techniques due to the high rate of heating that may be achieved [37,39]. Increasing the irradiation time has been shown to be an insignificant factor, as there was no improvement in the extraction efficiency [39]. In some cases, longer irradiation time can even decrease recovery of thermolabile compounds [37,40]. The extraction time required is mainly dependent on the ability

of the solvent or sample matrix to absorb microwave energy and transform it into thermal energy [39]. Another important factor that influences the extraction time is the number of samples or the total volume or mass of sample in the oven. Since the power generated is constant, it is distributed uniformly throughout the samples; if the number of samples increases, the power distributed to each sample will decrease [40]. With a decrease in power comes an increase in irradiation time in order to obtain the same yield [40].

1.7.6.5 Microwave Power

In general, the higher the microwave power, the shorter the exposure time; however, in some cases, high microwave power decreases the extraction efficiency, as it degrades the sample [37,39]. Therefore, the power applied should depend on the sample matrix itself and the number of samples undergoing simultaneous irradiation. The selection of microwave power can also depend on the solvent type. As a solvent's ability to absorb microwave energy increases, so does the temperature. In open-vessel systems, a rapid increase in temperature leads to rapid boiling, which in turns reduces the contact between the solvent and the sample, slowing the extraction process. In this instance, a lower microwave power is recommended [37,39].

1.7.6.6 Temperature

The optimal temperature for extraction depends on the nature of the matrix of the sample, the stability of the analyte and the extraction solvent [40]. For example, thermolabile compounds should not be extracted at high temperature. However, an advantage of high temperature is the

increase in analyte desorption and solvent penetration (wetting ability of solvent), resulting into a shorter extraction time [37].

1.7.6.7 Moisture

Water content is an important parameter in the extraction of compounds from plant matrices. Due to the polarity of water molecules, a rapid temperature increase can occur [40]. Thus, water within the matrix leads to direct heating of the matrix itself as opposed to heating of the matrix via solvent surrounding the sample. Similarly to increasing the solvent volume, an increase in matrix water content improves analyte recovery [40].

1.7.7 Influence of the Matrix Properties

The size of the sample particles greatly influences the recovery of compounds. As the particle size decreases, the surface area increases, allowing a greater contact between the solvent and the sample matrix, and consequently, a better extraction yield with further solvent and microwave penetration [37]. Complex matrices have a tendency to generate lower recoveries [39].

1.7.8 Instrumentation

Two types of microwave ovens exist: multimode and monomode (also called focused microwaves). In the multimode configuration, the microwave irradiation is essentially dispersed uniformly throughout the microwave cavity. In the single-mode microwave configuration, the microwave irradiation is focused on a restricted area of the sample, where a stronger electric field is applied [37,40]. Furthermore, microwave systems can be divided into two categories:

closed- and open-vessel systems. In both cases, the vessel material should be transparent to microwaves and inert to the solvent [37,40].

1.7.8.1 Closed-Vessel Systems

Since high temperatures (higher than the solvent's boiling point) and pressures are usually reached in closed-vessel systems, these parameters are monitored within the extraction vessel by detectors, to prevent overheating and over-pressurization. The vessel material, in these cases must also be thermally compatible.

Closed-vessel microwave systems have many advantages over the open-vessel microwave systems, mainly due to their ability to accommodate higher temperatures and pressures. This yields shorter extraction times and requires smaller solvent volumes, which in turn increases the analytical throughput and makes the technique more environmentally friendly and cost effective [37,40]. Also, a larger range of compounds can be extracted, including semi-volatile analytes, and the risk of atmospheric contamination and loss due to evaporation is eliminated [37,40]. Finally, the reproducibility and accuracy are also higher due to the ability to control the temperature and pressure [37].

Closed-vessel microwave extractions are limited by the cooling time required after every irradiation in order to avoid loss of volatile compounds [37]. Also, for safety reasons, addition or removal of solvent and reagent during the operation is not possible. The higher temperature and pressure also prevent the extraction of thermolabile analytes and pose safety risks [37,40]. In addition, a higher cost is associated with closed-vessel microwaves due to the instrument itself as

well as the specific vessel material required as result of the high temperatures and pressures attained [40].

1.7.8.2 Open-Vessel Systems

Open-vessel systems operate at atmospheric pressure, and therefore do not reach temperatures as high as in closed-vessels. In consequence, these systems require longer extraction times.

One of the main advantages of the open vessel microwave extraction is the increased safety due to lower pressure [37,40]. Since a lower temperature is generated, the samples do not require a cooling or depressurizing period after irradiation. This technique is also more suitable for thermolabile compounds and has a lower cost compared to closed-vessel microwave systems. Solvents and reagents can easily be added or removed at any time and the vessels can be made of various material compared to closed-vessel [37].

The limitations of open-vessel microwaves include longer extraction times as a result of lower temperatures attained and accounting for required pauses during extractions to limit extraction solvent boil-over [37]. In addition, a decrease in precision and lower yields compared to closed-vessel microwave systems are observed [37].

1.7.9 Advantages and Limitations of MAE

The main advantages of MAE are the significant reductions in extraction time and organic solvent consumption, mainly due to the high temperatures and pressures that may be

accommodated [30,34,36,37,39,40,46]. Similar observations were made with microwave digestion, clean-up and synthesis [28,43]. Compared to conventional extraction techniques, such as Soxhlet extraction, MAE can extract multiple samples simultaneously, increasing the throughput [30,34,36,46]. Microwave heating also offers homogenous heating throughout the sample, creating no temperature gradient due to the direct heating of the solvent or the sample matrix as opposed to the conduction/convection heating methods of standard passive extraction [40]. Today's instruments offer partial or complete automation during the analytical process which in turn provides better accuracy and precision [37]. MAE is also known to extract trace amounts of analyte compared to Soxhlet extraction [37]. Extraction yield and reproducibility are typically comparable or higher than those obtained by conventional extraction methods [37,40,46].

The drawbacks of MAE are the required cooling time for closed-vessel systems, the higher instrumental cost and the requirement of solvents and/or matrices that absorb microwave energy in order to generate heat [30].

1.7.10 MAE in Forensic Toxicology

MAE has been used in many different branches of chemistry, such as environmental analysis and medicinal chemistry and even the food industries, but its application to forensic toxicology is relatively new. Only a few studies have been published in this area. Franke et *al.* demonstrated an increase in recovery of drugs extracted from serum of 22 positive autopsy cases, using MAE with organic solvents [41]. Watterson and his group have published reports examining the utility of MAE for the extraction of drugs from ground bone [14,47]. Lastly, Fernández reported using

microwave energy for the simultaneous extraction of cocaine, CCE, BZE, 6AM, morphine and codeine from hair [46].

1.8 Ultrasound-Solvent Extraction

1.8.1 History and Applications

The first scientists to describe cavitation were Sir John Thornycroft and Sydney W. Barnaby in 1894, where strong vibrations and erosion were experienced and observed during the testing of a new high speed British Navy ship, The Destroyer, which lead to the formation of cavitation bubbles [48,49]. In 1927, Alfred L. Loomis was the first to study the unusual effects of sonochemistry; however, it was not until the 1980s that sonochemistry was further explored and articles were published where ultrasonic waves were used for mixing and homogenization purposes and compared to vortex mixing [50].

Today, ultrasounds are applied in many different areas including the medical field and industries for imaging, cleaning, vaporizing and also in home security alarms [46]. Most importantly, ultrasounds have been used in different aspects of chemistry for their physical and chemical effects. For example, ultrasounds have been used for reaction acceleration, degassing of liquids, slurry dispersion, homogenization, nebulisation, washing, derivatization and extraction [47,51,52,53,54,55,56].

1.8.2 Ultrasound and Cavitation Theory

Unlike electromagnetic waves, such as radio waves, infrared, visible, ultra-violet, X-rays and gamma rays, which can travel through vacuum, sound waves are mechanical vibrations that travel through matter. Ultrasonic waves are sound waves with a frequency range higher than the audible range of humans (16 Hz to 20 kHz), ranging from 20 KHz to 10 MHz [49,57]. Sound travels throughout matter in series of expansion and compression cycles, producing oscillation and promoting solvent penetration in samples. Throughout expansion cycles, the molecules are pulled apart, and are pushed together during compression cycles. When expansion occurs in a liquid, negative pressures are created at pre-existing weak points called cavities, where the vapour and dissolved gases migrate and accumulate forming bubbles. Once the negative pressure generated from the oscillation is greater than the tensile strength of the liquid, the bubble implodes and cavitation starts [46,47,49]. Cavitation is the rapid process (400 μs) of the formation and collapsing of bubbles during the oscillating expansion-compression cycles. It produces hot spots (~5000 °C) with a pressure of about 1000 atm due to the compression of gases and vapours [46,47,49,58,59]. When micro-bubbles implode, high temperatures and pressures are produced by gas and vapour compression that quickly dissipate through the bulk of the solvent, increasing the overall sample temperature only slightly and facilitating the analytes desorption and solvent penetration [47]. Occasionally, these extreme conditions can also lead to the formation of free radicals [49,56,57].

1.8.3 Cavitation Next to a Solid

In a liquid, the bubbles implode in a spherical shape due to the homogeneous surrounding. When cavitation occurs next to a solid, the formation and collapsing of a bubble is asymmetrical, since the surroundings are not uniform, leading to the formation of a high-speed liquid jet (400 km/h) [49]. When a bubble implodes, the potential energy from the expanded bubble transforms into kinetic energy and forms a liquid jet that penetrates the bubble [49,51]. The high-speed liquid jet penetrates the bubble from the opposite side that is in contact with the solid, towards the solid, in some cases fracturing the solid matrix, thus increasing the surface area and solvent penetration (figure 3) [49,51,58,60]. The implosion of these asymmetrical bubbles also causes turbulence and liquid circulation currents [60].

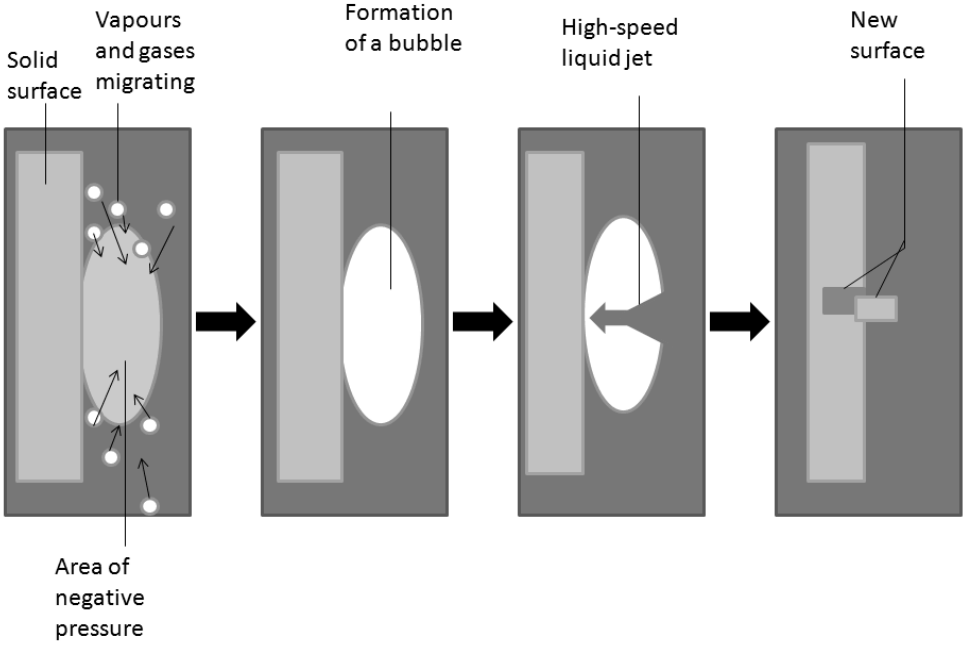


Figure 3: Cavitation in presence of a solid

1.8.4 Ultrasound Solvent Extraction

The physical effects produced by ultrasounic solvent extraction (USE) (turbulence and liquid circulation currents) increase the mass transfer and yield significantly [60]. USE is used as opposed to the traditional shaking method or Soxhlet extraction, as it provides a better contact between the sample and the solvent by increasing the solvent penetration which, in turn, reduces the extraction time and the volume of solvent required for maximum recovery [49,59,61]. It is also a good alternative technique for compounds that oxidize in acidic conditions and thermolabile analytes [51,61].

In environmental chemistry, ultrasound-solvent extraction has been used for the extraction of metallic analytes, pesticides, phenolic and aromatic compounds and natural products, from soils, sediments, powdered plant materials and foods [51,61].

1.8.5 Influential Parameters

Overall, the cavitation process is influenced by the extraction solvent, sample mass, solvent volume, extraction time, sample particle size, presence of dissolved gasses in the extracting solvent, medium temperature and intensity, and frequency of the applied ultrasonic waves (power density [W/cm²]) [49,51,58]. Other parameters that apply strictly to water baths are the presence of dissolved gases and vapours in the water of the bath, size and thickness of the vessel walls and position and number of samples in the bath [58]. Different extraction efficiencies of arsenic were observed when positioning the vessels at different places in the water bath and when comparing horizontal and vertical vessel positions by Capelo and his research group [58].

1.8.5.1 Extraction Solvent

The extraction solvent and its characteristics are some of the most important parameters of USE. The extraction efficiency depends on the ability of the solvent to extract the analytes from the solid and dissolve them, as well as its capacity to absorb and transmit the sound waves. The extraction solvent's surface tension and vapour pressure are important. As both of these characteristics increase, the cavitation intensity decreases [60]. Most extraction solvents cited in the literature for the extraction of a large variety of compounds include aqueous solvents, such as water and buffers and organic solvents, like methanol, ethanol, isopropanol, n-butanol, acetone, acetonitrile, ethyl acetate, hexane, dichloromethane and diethyl ether. The extraction solvent for USE is analyte dependent and is selected based on its viscosity and polarity, and on the chemical structure of the targeted compound [60,62].

1.8.5.2 Sample Mass and Solvent Volume

Samples require enough extraction solvent to immerse the matrix at all time. Capelo et *al.* observed no change in extraction efficiency with different solvent volume and sample mass as long as the mass-to-volume ratio was kept constant [58].

1.8.5.3 Extraction Time

The irradiation time required for maximum extraction efficiency depends mainly on the extraction solvent, the sample matrix and the power density generated. A greater amount of time is required when extracting analytes using a water bath compared to a probe [61]. In general, the higher the amplitude of the ultrasound irradiation, the shorter the extraction time required [58].

As the wave frequency is increased, the amplitude (power) must also be increased to keep the cavitation energy. Unlike probes, most water bath transducers do not have the ability to change the power density or frequency applied. The extraction time should be optimized for each analytes to ensure maximum extraction and prevent degradation of sensitive compounds [60,61].

1.8.5.4 Power Density

Ultrasonic systems usually operate at frequencies of 20, 40 or 80 kHz and power outputs of 50-700 W. As mentioned above, when increasing the frequency, power must also be increased in order to obtain the equivalent cavitation effect. Although bath systems usually function at a fixed frequency and power, these parameters can be adjusted in probe ultrasonicators, which are designed to deliver constant amplitude [58,60]. As the amplitude of the ultrasonic waves increases, so does the cavitation effect. Therefore, a larger power density generates more bubbles which, in turn, increase the extraction efficiency [62].

1.8.5.5 Sample Particle Size

The smaller the particles in a sample, the more homogeneous the sample will be, allowing a better distribution of the analyte through the matrix and therefore, better reproducibility. Smaller particles provide greater surface area, allowing a greater contact between the solid matrix and the extracting solvent, thus increasing analyte recovery [58]. However, the analyte distribution through the material must also be considered. If the analytes reside on the surface of the bone, the effect of particle size may be less relevant to the recovery as may be expected if they were uniformly distributed within the solid bone. The particle size depends greatly on the sample

matrix composition. On the other hand, Capelo et *al.* observed no significant difference in metal recovery from mussel tissue samples when comparing time exposed to ultrasound wave irradiation (10 and 120 min) and particle size (< 30 μm and > 300 μm) [58].

1.8.5.6 Medium Temperature

As a general rule, when the temperature increases, so does analyte extraction and dissolution. However, temperature must also be optimized in order to prevent compound degradation.

1.8.6 Instrumentation

1.8.6.1 Bath

A piezoelectric transducer installed in a water bath can create a power density between 1-5 W/cm². The amount of energy transferred to the sample depends on the dimension of the bath, water volume, the position of the transducer, the material and the thickness of the vessel walls, as well as the position and number of samples in the bath [51]. For this reason, comparison of results obtained from different laboratories is almost impossible.

The main advantages of ultrasonic baths are their low cost (approximately $1000 US) compared to probes ($2000-4500 US) and their higher throughput. For these reasons, ultrasonic baths are widely used. In addition, due to the simplicity of the technique, vessels of many different shapes and materials can be accommodated [58]. Some of the disadvantages of water baths, that affect the reproducibility, are the lack of uniformity in the distribution of the ultrasounds energy and the decline in power over time [49,51,58]. The energy generated by the bath water transducer

first has to go through the water of the bath and then the vessel walls in order to attain the sample; therefore energy is lost at each step which results in less cavitation [49].

1.8.6.2 Probe

It is well known that probes and horns can generate much more powerful ultrasonic vibrations (50-750 W/cm²) [49]. In this situation, the ultrasounds produced are transferred to a metal rod, usually made of titanium, which is immersed directly into the sample, preventing energy loss by water and vessel wall absorption. Due to the focused ultrasound energy, which generates greater cavitation, shorter extraction times are required and a generally higher reproducibility and analyte recovery is observed [49,51,58]. Furthermore, the amplitude and frequency can usually be modified [51]. Probes are limited by their high cost and their lower sample throughput [58].

1.8.7 Advantages and Limitations of USE

USE is a simple and inexpensive substitute technique to conventional extraction methods with high extraction efficiencies and yield. This technique requires less extraction solvent and time, making it an eco-friendly and high throughput technique [49,58,59,61]. USE is also a less aggressive technique compared to MAE and Soxhlet extraction since it operates at a low temperature and pressure. Molecular decomposition of sensitive and thermolabile compounds is thus minimized [49,59,60,61,62]. Also, unlike MAE, USE is not limited by the extraction solvent [49].

The major drawback of USE is the lack of reproducibility from ultrasonication water baths, especially between instruments, since the generation and distribution of the ultrasound energy is not constant [49,58,61].

1.8.8 USE in Forensic Toxicology

Ultrasonic solvent extraction is a relatively new application to forensic toxicology, and one that has not been applied to the extraction of drugs and drug metabolites from bone. In the past, USE has been employed to isolate pesticides from soil samples and for the extraction of drugs from hair [63,64].

1.9 Drugs of Study

Drugs most commonly found in forensic toxicological cases include recreational, illicit and therapeutic drugs. These drugs include caffeine, nicotine, alcohols (ex: ethanol), cannabinoids (ex: marijuana and hashish), opioids (ex: heroin, codeine, morphine and opium), stimulants (ex: cocaine, amphetamine), benzodiazepines (ex: diazepam), antipsychotics (ex: chlorpromazine), antidepressants (ex: amitriptyline and citalopram) and many more [1,2,3,16 ,65].

1.9.1 Amitriptyline and Nortriptyline

Amitriptyline (AMI) is a tricyclic antidepressant (TCA), and is part of the first generation antidepressants. It was released for clinical use in 1961, and is typically used to treat depression,

anxiety disorders, eating disorders and attention deficit hyperactivity disorder (ADHD) [66,67,68,69,70,71,72]. AMI has also been used to treat nocturia and enuresis in children and more recently, AMI has also been found effective in reducing neuropathic pain, fibromyalgia, headaches and severe chronic low-back pain [67,68,73]. TCAs are characterized by the three hydrocarbon rings structure linked to an alkylamine chain containing a tertiary or secondary amino group at the terminus (figure 4) [66].

AMI, 3-(10,11-dibenzo[a,d]-cycloheptene-5-ylidene)-*N,N*-dimethyl-1-propamine, blocks the reuptake of norepinephrine, dopamine and serotonin neurotransmitters associated with the regulation of mood and anxiety [18]. AMI is administered orally or intramuscularly (IM); doses vary from 25-150 mg for outpatients and up to 300 mg for hospitalized patients [74]. At therapeutic concentrations, side effects include anticholinergic effects such as dry mouth, blurred vision, drowsiness, sedation, lowered blood pressure, weight gain, sweating, and fatigue. Toxic symptoms include cardiac dysrhythmia, severe hypotension, stomach and digestive problems, convulsions and central nervous system (CNS) depression [74,75]. Therapeutic and toxic blood concentrations in humans are reported in table 2.

In humans, AMI gets metabolized through cytochrome P450 (CYP) enzymes in the liver into more polar metabolites. CYP2C19 is the main enzyme responsible for the N-desmethylation of AMI to nortriptyline (NTRIP), an active metabolite, while CYP2D6 is responsible for the formation of the hydroxyl metabolites (10-hydroxyamitriptyline, 10-hydroxynortriptyline and 10-hydroxydinortriptyline) [1,66,75,76]. AMI's half-life ranges from 8 to 51 h in humans; the metabolic pathway is shown in figure 4 [74].

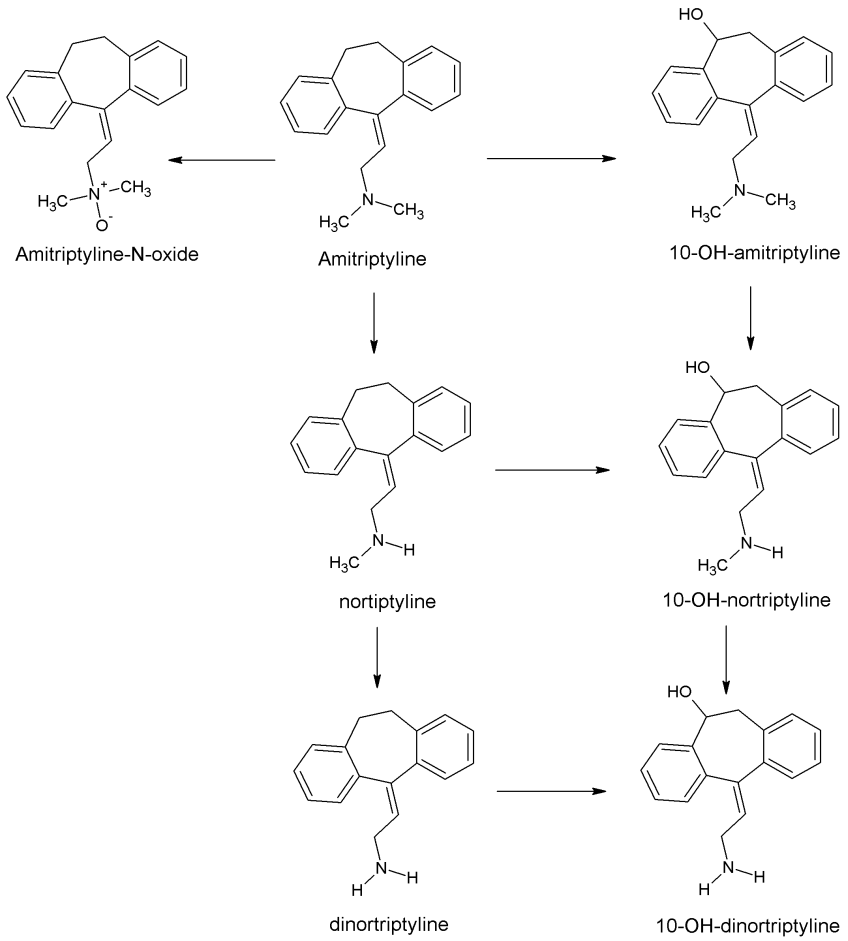


Figure 4: Metabolic pathway of amitriptyline and nortriptyline in human [74].

Due to its active pharmacodynamic effects nortriptyline, 3-(10,11-dibenzo[a,d]-cycloheptene-5-ylidene-*N*-methyl-1-propamine, on its own, is also marketed as an antidepressant [74,78]. The pharmacokinetics and pharmacodymanics of NTRIP are similar to those of AMI. Neurotransmitter reuptake is inhibited, allowing them to accumulate in the intracellular space [66]. NTRIP gets further metabolized by N-desmethylation (CYP2C19) and 10-hydroxylation (CYP2D6).

Table 2: Pharmacodynamics and pharmacokinetics of selected drugs [74]

Drugs	Amitriptyline	Nortriptyline	Citalopram	Desmethylcitalopram	Pentobarbital
Brand names	Elavil, Amitid, Endep, Amitril	Aventyl, Sensoval, Pamelor, Norpress	Celexa, Cipramil	N/A	Nembutal, Dorsital, Pentobarbitone
Tablets (mg) and solutions (mg/mL) available	Tablets: 10, 25, 50, 75, 100 and 150 Solution: 10	Capsules: 10, 25, 50 and 75 Syrup: 2	Tablets: 10, 20 and 40 Solution: 2	N/A	Tablets: 15-200 Solution: 50
Route of administration	Oral, IM	Oral, IM	Oral,	N/A	Oral, IM, rectal
Dose range (mg)	25-150 (300 hospitalized patients)	25-150	10-50	N/A	15-200 (IM)
Therapeutic blood conc. (mg/L)	0.016-0.242	0.014-0.180	0.05-0.4	N/A	1-5
Side effects	Anticholinergic effects: dry mouth, blurred vision, drowsiness, sedation, lowered blood pressure, weight gain sweating, fatigue		Drowsiness, insomnia, nausea, weight changes, frequent urination, decreased sex drive, anorgasmia, dry mouth, fatigue		Sedation, drowsiness, lethargy, nausea, vertigo, vomiting
Toxic blood conc. (mg/L)	> 0.5	> 0.5	> 3	N/A	> 10
	> 1.0 (combined)				
Toxic symptoms	Cardiac dysrythmia, severe hypotension, stomach and digestive problems, convulsions, CNS depression		Somnolence, nausea, vomiting, nystagmus, dilated pupils, sweating, tremor, tachycardia, migraine, diarrhea, insomnia, hypotension		Drowsiness, confusion, respiratory depression, cardiovascular depression
Half life (h)	8-51	15-90	25-40	N/A	20-30
Bioavailability (%)	30-60	30-70	80	N/A	70-90
Volume of distribution (L/kg)	6-10	20-57	12-16	N/A	0.5-1
Protein binding (%)	> 90	90	70-80	N/A	20-60
pKa	9.4	9.7	9.5	N/A	7.9
References	66-76,81,82		74,77,78		74

Due to its ubiquitous use and narrow therapeutic range, amitriptyline is the most encountered TCA in emergency toxicology screening, drug abuse testing and forensic medical examination [1,2,66,72]. In addition, drug interactions occur when other drugs with CNS depressant

properties, such as ethanol, are taken simultaneously with AMI, causing symptoms from mild impairment to respiratory depression, reduced heart rate, coma and death. Toxicity arises when ethanol is co-administered, inhibiting P450 enzymes, prolonging the half-life of the drug, increasing the AMI plasma concentration during the absorption and distribution phase, and preventing elimination [75]. Other possible drug interactions include CYP450 inducers and inhibitors, and mono-amine oxidase inhibitors (MAOI). Today TCAs have mainly been replaced by newer and safer antidepressants with less severe side effect and lower risks of cardiovascular and neuronal toxicity [66,68].

1.9.2 Citalopram and Desmethylcitalopram

Citalopram (CIT) is part of the third generation antidepressants, the selective serotonin reuptake inhibitors (SSRIs), and has been used clinically since the mid 1980s to treat depression, obsessive-compulsive disorder (OCD), panic disorder, anxiety, post-traumatic stress disorder, smoking cessation and ethanol abuse [19,69,77,78]. CIT, (RS)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile, is structurally unrelated to other SSRIs and is available as a racemic mixture of *R*- and *S*- enantiomers (figure 5), but *S*-CIT is mainly responsible for the SSRI effect, and is now marketed as a single enantiomer drug, escitalopram [74,77,78,79]. As the name suggests, SSRIs inhibit the reuptake of serotonin from the synaptic cleft to the presynaptic neurons, allowing the postsynaptic neurons to be stimulated longer, but has little effect on noradrenaline and dopamine [78]. CIT is administered orally, with doses varying from 10-50 mg, and typical blood concentrations of 0.04 - 0.1 mg/L [74]. Mild side effects include drowsiness, nausea, frequent urination, dry mouth, and decreased sex drive,

while toxic blood concentrations cause somnolence, nausea, vomiting, dilated pupils, tachycardia and hypotension – see table 2 for therapeutic and fatal blood concentration range [74].

Through CYP2C19, CYP3A4 and CYP2C6, citalopram undergoes mono- and di-N-demethylation to desmethylcitalopram (DCIT) and didesmethylcitalopram (DDCIT) (figure 5). Both are pharmacologically active, but less potent than the parent drug, slightly prolonging the neuronal activity [74,79]. CIT has a half-life ranging from 25 to 40 h in humans [74].

Drug interactions between SSRIs and MAOI can occur by creating an excess of serotonergic activity at the CNS and peripheral serotonin receptors. Symptoms of serotonin toxicity include increased heart rate, sweating, hyperactive bowel sounds, high blood pressure and hyperthermia. The possibilities of drug interactions can occur through many different mechanisms that are hepatically metabolized. In addition, the long elimination half-life increases the potential interaction with other drugs even after use has been discontinued [77,78].

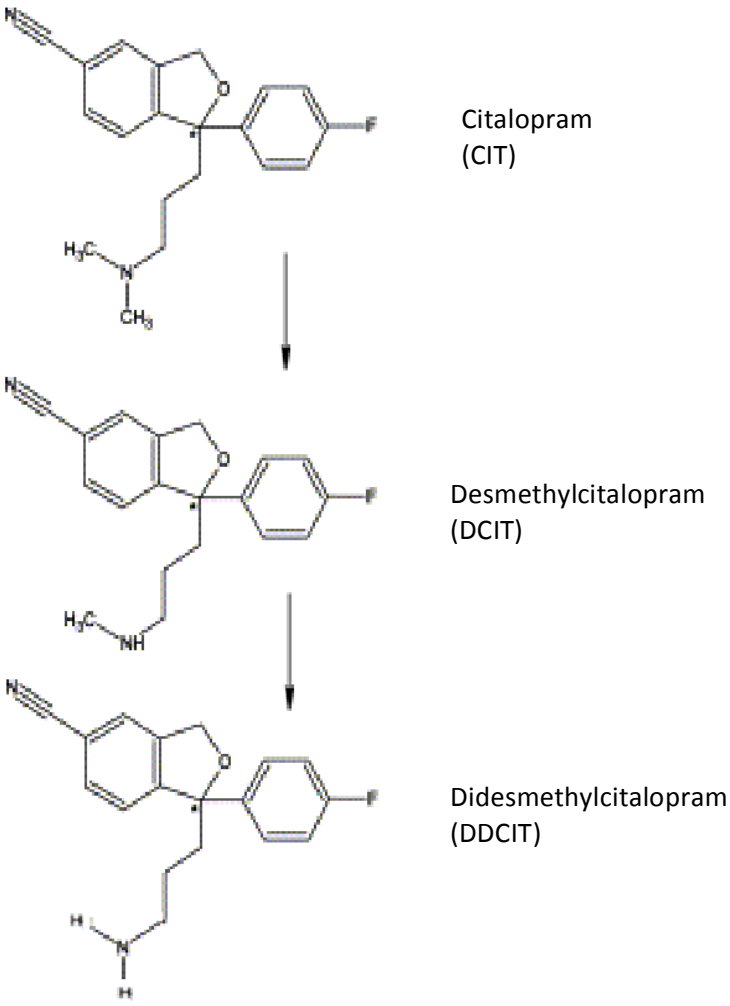


Figure 5: Metabolic pathway of citalopram and desmethylcitalopram [74].

1.9.3 Pentobarbital

Pentobarbital (PB) is a short acting barbiturate, first introduced in the early 1930s. It is used as a sedative, hypnotic, anticonvulsant for epilepsy, anxiolytic (antipanic or antianxiety agent) and veterinary anesthetic and euthanasia agent [74,80,81]. PB was also once used for execution of humans in the United States, and to treat insomnia and anxiety, but has now been replaced by benzodiazepines due to its low therapeutic index and potential for abuse [81]. On occasion,

pentobarbital is also used to reduce intracranial pressure, and induce coma in cerebral ischemia patients and assisted suicide [80,82].

Pentobarbital, 5-ethyl-5-(pentan-2-yl)-1,3-diazinane-2,4,6-trione, acts as a non-selective CNS depressant (figure 6). The mechanism of action is still not completely understood, but it acts by depressing the CNS and inhibiting certain nerve response centres [74,80,82,81]. PB is usually administered orally (15-200 mg) and side effects include loss of balance or coordination, nausea and headache. As the dose increases, so does the depression of the brain functions; first signs of toxicity observed are confusion, shallow breathing and slow heart rate. See table 2 for therapeutic and fatal blood concentration range [74].

PB has a half-life of 20-30 h in humans and undergoes first pass metabolism in the liver. Its primary biotransformation is via oxidation into a diastereomeric mixture of alcohols (inert metabolites) and secondarily by N-hydroxylation [74,81,83,84,85].

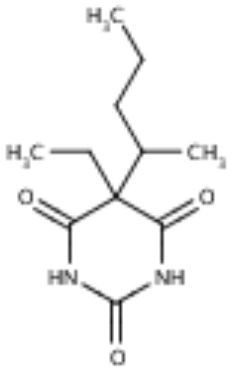


Figure 6: Chemical structure of pentobarbital [74].

Drug interactions with barbiturates occur with other CNS depressants, such as ethanol, opioids, antihistamines, and sedative/hypnotic agents, where additive effects result in slower brain activities, cardiovascular instabilities and respiratory depression [86].

1.10 Preparation of Solid Samples

Many techniques have been used for the detection and quantification of these five drugs in the past, but prior to analysis, sample preparation, such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE), is required to clean up and reduce the matrix effects created from its complex biological matrix [18,67,69,72,76,77,78,86,87]. While LLE used to be the traditional sample preparation method, SPE is now predominant in the literature.

Two types of SPE columns have been used to extract these drugs. Reversed-phase SPE is the least selective mechanism and will retain most molecules with any hydrophobic character by Van der Waals forces and dipole-dipole interactions, while mixed-mode columns retain substances by both hydrophobic and electrostatic interactions [19,69,87,88,89]. The literature shows a tendency towards mixed-mode columns because of its higher selectivity and the ability to extract multiple analytes with different chemical properties (acidic, neutral and basic) all in one column. Using this type of column, at pH 6, weakly acidic compounds, such as pentobarbital, remain non-ionized and are retained to the column by hydrophobic interactions. On the other hand, strongly basic compounds, such as AMI, NTRIP, CIT and DCIT, are protonated and retained by electrostatic interactions [87]. The column can then be washed with mixtures of aqueous and water-miscible organic solvents at low pH to prevent the loss of basic

compounds during the first elution. At this point, PB can be eluted with a polar solvent and subsequently the antidepressants are eluted by increasing the pH with strong NH4OH, rendering them neutral, along with a less polar organic solvent [87].

1.11 Analysis of DCIT, CIT, NTRIP, AMI and PB

The identification of these five analytes has been performed by many different methods including immunoassay [19,66,68], liquid chromatography with a diode array detector (HPLC-DAD) [66,67,68,71,72,78], or mass spectrometry (LC-MS) [66,72] and gas chromatography (GC) with electron impact (EI) or chemical ionization (CI) and MS [19,66,67,72,78], flame ionization detector (FID) [19,48,90], or nitrogen-phosphorous detector (NPD) [19,69,91].

1.12 Use of Experimental Animals in Forensic Toxicology Research

The use of human tissue for forensic research is often impossible for legal reasons. The only way human tissue can be acquired is through autopsy, in which important information such as history of drug use and dose are unknown. In addition, due to legal restrictions, using autopsy tissue in research could cause problems in some jurisdictions. For these reasons, animal models are used. Important variables such as the drug exposure, dose, route of administration, delay between drug administration and death, and post-mortem circumstances can be controlled. Furthermore, drug properties like distribution between tissue types, accumulation and analytical method development can easily be understood and developed using animal models. The disadvantage is its limited applicability to human cases.

1.13 Stability of Drugs in Bone during Decomposition

Because of the chemical, biological and physical changes occurring to a corpse during putrefaction, forensic toxicologists must consider the possibility of drug degradation. During putrefaction, various changes in bone occurs, including exchange of ions, uptake of circulating organics and microbiological attack; in turn, this results in the breakdown of collagen and alteration and leaching of the mineral matrix [92]. In addition, bone damage, such as ante-mortem trauma or post-mortem animal scavenging, can increase bone porosity, leaving the drugs and metabolites more vulnerable to environmental factors and biochemical aspects, such as evaporation, release from binding site and drug or protein degradation [10].

Unfortunately, little information is available in the literature about drug stability in skeletal tissue, but many reports show the detection of a variety of drugs from skeletal tissue after long periods of decomposition in outdoor conditions or burial. McIntyre et *al.* [2] were able to detect 12 different analytes, covering a wide variety of drugs, from 36 cases, using the fresh mid-femoral bone. Drugs detected include antidepressants like mianserin, moclobemide, sertraline and TCAs (amitriptyline, doxepin and dothiepine), antipsychotics such as chlorpromazine, thioridazine and clozapine, and benzodiazepines like diazepam, oxazepam and temazepam [2]. Levels of diacetylmorphine and its metabolites, 6-acethylmorphine and morphine, from fresh mice bone were compared to those buried in open air at room temperature for 2 months, with no significant difference observed in analyte recovery [3]. Midazolam was detected in buried mice bone and diazepam and its metabolite nordiazepam were observed in fresh rat bone, as well as skeletonised porcine bone that was left outdoors for 2 years [65,13,14,93]. In contrast, a paper reports 54 % morphine loss in human thigh bone after 1 year burial [16]. Other drugs such as

methamphetamine and amphetamine were detected in bone stored for 2 years in open air and in rabbit bone after 2 years of storage in tap water at room temperature, while aminopyrine was detected in remains estimated to be 2-5 years old [3,15,94]. It appears drug stability in decomposing skeletal tissue may be drug dependent.

It has been demonstrated by many that drug detection from this unusual tissue is possible, but that the interpretation of analytical results remains uncertain. Cases where bone would be used as samples for toxicological analysis would often have no conventional fluids or tissue available. For this reason, research of drug deposition and analysis from bone should also be performed on nearly completly skeletonised remains, as opposed to fresh bone [47].

1.14 Goal of Research

For the purpose of this research, the bones of a pig (*Sus domestica*) were donated from the Franklin County Coroner's Office in Ohio. The original research investigated the temporal fate of drugs in decomposing porcine tissue [95]. Pigs were selected due to their resemblance in digestive and cardiovascular physiology to humans, and their comparable size. The original study was performed in accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals" and was approved by the ILACUC, The Ohio State University, Columbus, OH [95].

The main goal of this research was to characterize and optimize new assisted extraction methods (microwave-assisted extraction and ultrasonic solvent extraction) for the extraction of selected drugs and metabolites from skeletal tissue, and compare them with the standard extraction

method, passive extraction. Samples were analyzed by gas chromatography-mass spectrometry or ultra performance liquid chromatography with a diode array detector. The effect of different variables on the extraction yield and rate was examined, including the effect of extraction solvent, sample mass, extraction time, solvent volume, microwave power and the effect of the presence of gas in solutions undergoing ultrasonication. In addition, other steps in sample preparation were optimized: different solutions and times were tested for protein and lipid precipitation and different columns as well as column washes were tested for SPE.

Chapter 2

2 Material and Methods

2.1 Chemicals

Drug standards (Cerilliant, Round Rock, TX) were obtained as 1 mg/mL methanolic solutions and diluted as required. Trimethylphenyl ammonium hydroxide (TMPAH) was purchased from United Chemical Technologies (Bristol, PA). Methanol (MeOH) and ethyl acetate (EA), used in drug extraction, were reagent grade and purchased from EMD chemicals (Gibbstown, NJ). Acetonitrile (ACN), used in UPLC-DAD analysis, was HPLC grade and purchased from EMD chemicals (Gibbstown, NJ). All other chemicals were reagent grade and obtained from EMD chemicals (Gibbstown, NJ).

2.2 Animals and Drug Administration

Vertebral bone of a Yorkshire/Hampshire cross-breed pig used in a previous study investigating the temporal fate of drugs in decomposing porcine tissue was used throughout this work [95]. Pigs for the study were purchased from Kidron Auction (Kidron, Ohio) and weighed between 120 and 180 pounds (55 and 82 kg). The drug cocktail administered (by gavage) to this particular pig contained amitriptyline (75 mg/kg), citalopram (7 mg/kg), diazepam (7.5 mg/kg) and morphine (0.8 mg/kg). Drug reference standards and drug cocktail preparation can be found in the original report [95]. The animals were anaesthetized with pentobarbital (30 mg/kg, IP) 4 hours after drug administration. Once sedated, the pigs were sacrificed with 10 mL Beuthanasia-

D® (390 mg/mL sodium pentobarbital and 50 mg/mL sodium phenytoin (IC) Schering-Plough Animal Health, Union, NJ) [95].

2.3 Bone Preparation

Pig remains were left to decompose in a secure rural location in Ohio for approximately 2 years before the collection of skeletal tissues. Bones were separated according to their anatomical location. Drug-free porcine bone, donated by Costco Wholesale Corporation, was used as matrix simulation for the stability study and negative controls. Control bones were buried in biologically active soil (Selection Garden Soil enriched with Compost) for approximately 1 month (in an air tight container) and further boiled for approximately 40 hours in water in order to remove as much fatty tissues as possible. Both control and drug positive bones were washed with distilled water, methanol and acetone (10 mL) to remove soil and other surface contaminants; wash solutions were not analyzed. Bones were then crushed manually in a plastic bag with a mallet prior to being ground to powder with a domestic grinder (figure 7). Samples were analyzed for amitriptyline (AMI), nortriptyline (NTRIP), citalopram, (CIT), desmethylcitalopram (DCIT), and pentobarbital (PB).

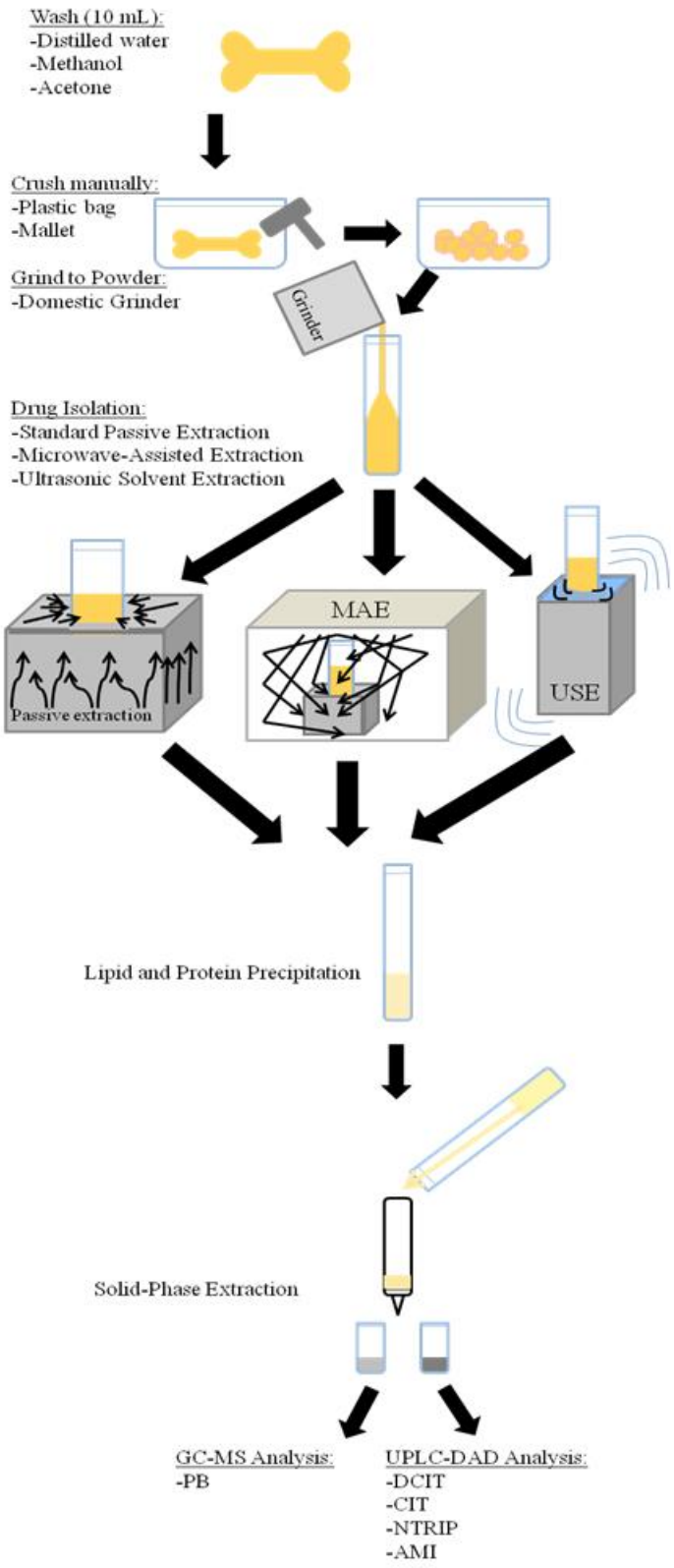


Figure 7: Steps in the sample preparation for the extraction of select drugs and metabolites from skeletal tissue

Prior to optimizing the solvent extraction steps, the protein and lipid precipitation technique, along with the SPE, were optimized by examining the effect of different precipitation solvent, storage time, SPE column, and columns washes on the analyte recovery.

2.4 Optimization of Solid-Phase Extraction (SPE)

2.4.1 SPE Column Comparison

The influence of different SPE columns on the extraction yield was investigated. Samples (n = 2) spiked with 100 ng/mL of PB and the antidepressants, as well as a negative control (n = 1), were made of matrix-matched bone extract, also called bone tissue extract (BTE, 1 mL), a solution of decomposed soft tissue and extracted bone homogenized in phosphate buffered saline at pH 6 (PBS6). Based on the results from the protein and lipid precipitation section, PBS6 (0.1 M, pH 6, 3 mL) and ACN:MeOH (1:1 v/v 3 mL) was added before samples were vortexed and stored at -20 °C for 1 h. Samples were centrifuged (4000 RPM, 10 min) and supernatant was removed and evaporated to approximately 1 mL under a gentle stream of air at 70 °C. Samples were diluted to 4 mL with PBS6 and acidified with 200 µL of glacial acetic acid before loading.

Samples were extracted with CleanScreen CSDAU mixed-mode columns (200 mg, United Chemical Technologies, Bristol, PA), CleanScreen XCEL 1 columns (130 mg, United Chemical Technologies, Bristol, PA) or Strata XC columns (60 mg, Phenomenex Inc., Torrance, CA). Columns were conditioned with methanol, distilled water and PBS6 (3 mL). Samples were then loaded by gravity and columns were sequentially washed with PBS6 (3 mL) and acetic acid (0.1

M, 3 mL). Columns were dried under vacuum (10 in Hg, 5 min) before a final methanol wash (3 mL), where PB was eluted and collected. Columns were then dried under vacuum (15 inHg, 10 min) and DCIT, CIT, NTRIP and AMI were eluted using a mixture of ammonium hydroxide:isopropanol:EA (3:17:80, 2 x 3 mL). All extracts were evaporated to complete dryness under a gentle stream of air at 70 °C.

2.4.2 Effect of Hexane Wash

Based on the evaluation of the different SPE columns, a second set of extractions was undertaken to establish the influence of a hexane wash step during the extraction on the level of interfering compounds directly affecting the extraction yield. Samples (n = 2) containing 100 ng/mL of PB and antidepressants, and negative control (n = 1), were made of BTE (1 mL). Samples were treated as mentioned in *2.4.1 SPE column comparison* section for the precipitation of proteins and lipids. Samples were centrifuged (4000 RPM, 10 min) and supernatant was removed and evaporated to approximately 1 mL under a gentle stream of air at 70 °C. Samples were diluted to 4 mL with PBS6 and acidified with 200 µL of glacial acetic acid before loading.

Samples were extracted with CleanScreen CSDAU mixed-mode columns (200 mg, United Chemical Technologies, Bristol, PA), based on the results of the SPE columns comparison. Half the samples were extracted as mentioned in the previous section, while the other half were conditioned and loaded the same way, but were washed with PBS6 (3 mL) and acetic acid (0.1 M, 3 mL) before they underwent vacuum drying (10 inHg, 5 min). Columns were sequentially washed with hexane (3 mL) and dried under vacuum (15 inHg, 10 min) before a final methanol wash (3 mL), where PB was eluted and collected. Columns were then dried under vacuum dried

(15 inHg, 10 min) and antidepressants were eluted using a mixture of ammonium hydroxide:isopropanol:EA (3:17:80, v/v/v, 2 x 3 mL). All extracts were evaporated to complete dryness under a gentle stream of air at 70 °C.

2.5 Optimization of Protein and Lipid Precipitation

2.5.1 Effect of Solvent

The influence of different protein and lipid precipitation solutions [acetonitrile (ACN):MeOH (1:1 v/v), ACN, acetone: MeOH (1:1 v/v) and acetone] on the extraction yield was investigated. Samples (n = 3) containing 100 ng/mL of CIT, DCIT, AMI, NTRIP and PB, and a negative control (n = 1), were made of BTE (1 mL). A total of 16 samples were prepared. Secobarbital (SB, 50 ng) and desipramine (DMI, 250 ng) were added as internal standards (ISTDs) for PB and the antidepressants, respectively. PBS6 (0.1 M, pH 6, 3 mL) and one of the different precipitation solutions (3 mL) were added before samples were vortexed and stored at -20 °C for 1 h to precipitate proteins and lipids.

2.5.2 Effect of Time

Based on the results of solvents during the protein and lipid precipitation, a second set of extractions was undertaken. The influence of different storage times at -20 °C to precipitate proteins and lipids on the extraction yield was investigated. Two drug concentration sets were used: PB, CIT and DCIT were observed at 50 and 1000 ng/mL, and AMI and NTRIP were observed at 100 and 2500 ng/mL (low and high concentrations, respectively). Samples (n = 3)

and negative control (n = 1) were made of BTE (1 mL). A total of 16 samples were prepared. PBS6 (0.1 M, pH 6, 3 mL), ACN:MeOH (1:1, v/v, 3 mL) and ISTDs were added before samples were vortexed and stored at -20 °C for 1 or 24 h of incubation to precipitate proteins and lipids.

2.6 SPE Method Validation – Precision and Linearity

The precision of the analyte response ratio (RR) was measured as the coefficient of variation (% CV):

$$RR = \frac{\text{Peak Area Analyte}}{\text{Peak Area ISTD}}$$

$$\% \text{ CV} = \left[\frac{\text{Standard Deviation}}{\text{Mean}}\right] \text{x } 100$$

Analytes were measured in replicates (n = 8) from spiked BTE, in order to determine method precision.

The range of concentrations assayed was 5-2000 ng/mL for PB, DCIT and CIT and 5-5000 ng/mL for NTRIP and AMI. Based on the SPE protocol used, where the final sample volume immediately prior to SPE is 1 mL, these ranges correspond to 5-2000 ng/g and 5-5000 ng/g, respectively. All points on curves were prepared in 1 mL of BTE. Samples then underwent lipid and protein precipitation and SPE as mentioned above. The limit of quantitation (LOQ) was determined as the lowest concentration following validation criteria of precision (% CV smaller than 20) and accuracy and the cut off level was the lowest concentration assayed that did not

meet those criteria while still detecting the analytes. The cut off level and LOQ were determined with replicates (n = 9) of 5, 10 and 25 ng/mL made from drug free BTE.

2.7 Drug Stability

The stability of all five drugs was examined, for passive, microwave and ultrasonic extraction, in both MeOH and EA. Samples containing the set of analytes at one of two different concentrations in 10 mL of solvent were added to control bone (2 g). The samples then underwent passive incubation, microwave irradiation or ultrasonication. Two drug concentration sets were used for each analyte: PB, CIT and DCIT at 200 and 2000 ng/mL, and AMI and NTRIP at 500 and 5000 ng/mL (low-to-mid and high concentrations, respectively). These concentrations were chosen based on prior extractions made from samples of the same vertebral bone [93], where higher concentrations of AMI and NTRIP were observed. Samples (n = 3) were weighed in screw-cap test tubes and a drug-free sample was included as quality control, for a total of 42 samples for all extraction methods. The response ratio was used to determine the stability of each drug, when comparing the RR of a sample exposed to the extraction method after different time periods.

2.7.1 Standard Passive Extraction

Samples undergoing passive extraction were vortexed and incubated on a hot plate at 50 °C (± 1-2 °C) for a total of 96 h. Every 24 h, 1 mL of solvent was removed from each sample and evaporated to complete dryness under a gentle stream of air at 70 °C.

2.7.2 Microwave-Assisted Extraction (MAE)

Samples prepared for MAE were vortexed before microwave irradiation in a household Danby microwave oven DMW1153W (1100 W, 2450 MHz) equipped with a turntable for a total of 30 min in successive intervals of 10 s. All test tubes were manually stirred between 10 s irradiation interval to ensure mixing of solvent, to increase the surface area the solvent had in contact with the bone and to release any dissolved gases in the order to prevent boiling. A volume of 1 mL was removed from each sample at pre-determined times (3, 6, 9, 12, 15, 21 and 30 min) and evaporated to complete dryness under a gentle stream of air at 70 °C.

2.7.3 Ultrasound Solvent Extraction (USE)

Samples to be extracted by USE were first vortexed prior to sonication in a bath sonicator (VWR 150T Aquastar, VWR Canlab, Mississauga, ON) for a total of 90 min. A volume of 1 mL was removed from each sample at pre-determined times (10, 30, 45, 60 and 90 min) and bath water was also changed every 30 min in order to reduce the influence of bath heating on the extraction process. Samples were treated in the same manner as indicated above.

2.8 Optimization/Characterization of Drugs Extraction from Bone

2.8.1 Effects of Solvents, Sample Mass and Extraction Time

Drug extraction was performed by adding 10 mL of solvent (MeOH or EA) to 1 or 2 g (n = 3) of ground vertebrae. A drug-free (n = 1) sample of porcine bone was also weighed into a screw-cap

glass test tube for each weight and solvent, for quality control. A total of 64 samples were weighed, where 32 were incubated (16 in each short and long passive incubation intervals), 16 were irradiated and 16 were sonicated under the same conditions as described above.

2.8.1.1 Standard Passive Extraction

Samples extracted passively were divided into two groups based on the incubation time – long term intervals and short term intervals. All samples extracted passively were incubated for a predetermined period of time after which the supernatant solvent was recovered, bones were sequentially washed with two 5 mL of the same solvent before a new 10 mL was added in order to monitor the rate of extraction. The washes were pooled with the initial supernatant; samples were then completely evaporated at 70 °C under a gentle stream of air. Samples submitted to long interval passive extraction were incubated for a total of 96 h, where the solvent was removed and bones were washed every 24 h. The samples submitted to the shorter term of passive incubation were recovered and washed after 3, 6, 10, 15, 21, 30, 45 min, 1, 1.5, 3, 6, 12, 18, and 24 h.

2.8.1.2 Microwave-Assisted Extraction

Samples extracted by MAE were irradiated for a total of 30 min. After 3, 6, 9, 12, 15, 21, and 30 min, the solvent was removed and samples were treated in the same manner as the samples extracted passively.

2.8.1.3 Ultrasound Solvent Extraction

Samples extracted by USE were submitted to ultrasonic agitation for a total of 90 min. After 10, 30, 45, 60 and 90 min, the solvent was removed and samples were treated in the same manner as the samples extracted passively and by microwave irradiation.

2.8.2 Effect of Solvent Volume

Based on the results of experiments examining the influence of the extraction solvent, sample mass and extraction time on the extraction yield, a second set of extractions was undertaken to investigate the influence of solvent volume on the analyte recovery. Pig bone (1 g, n = 3) and control bone (1 g, n = 1) were weighed into screw-cap glass test tubes and 5, 10 or 15 mL of methanol was added before passive incubation, irradiation or sonication. A total of 36 samples were prepared.

The passive incubation, irradiation, sonication and solvent recovery was performed in the same manner as mentioned in the *2.8.1 Effect of Solvent, Sample Mass and Extraction Time* section, but the samples were incubated for a total of 72 h, irradiated for 15 min or sonicated for a total of 45 min only.

2.8.3 Effect of Microwave Power

The influence of microwave power on the extraction yield was investigated. This extraction set was based on the evaluation of the extraction solvent, sample mass, extraction time, and solvent volume. Samples of pig bone (1 g, n = 3) and control bone (1 g, n = 1) were weighted into screw-

cap test tubes and 5 mL of methanol was added before microwave irradiation. A total of 16 samples were prepared.

Samples were irradiated for a total of 15 min at a percent power of 100, 80, 50 or 20 %. Solvent was recovered and samples were treated in the same method as mentioned in the two previous sections.

2.8.4 Effect of Presence of Gas in Water Bath and Extraction Solvent

Based on the results of experiments examining the influence of the extraction solvent, sample mass, extraction time, and extraction solvent volume, a fourth set of extractions was undertaken, where the influence of dissolved gasses in bath water and extraction solvent on the extraction yield was investigated. The bath water of the sonicator and methanol (extraction solvent) were degassed by pressure reduction (vacuum 15 inHg, 10 min). Pig bone (1 g, n = 3) and control (1 g, n = 1) were weighed in screw-cap test tubes and 5 mL of methanol (either subject to degasification or not) was added before ultrasonic agitation. A total of 16 samples were prepared. Table 3 demonstrates the four conditions the samples were subjected to. Solvent was recovered and samples were treated in the same manner as mentioned in the section *2.8.1 Effect of Solvent, Sample Mass and Extraction Time*, but the samples were sonicated for a total of 45 min only.

Table 3: Conditions for samples subjected to USE, investigating the effects of presence of gas in water bath and extraction solvent

Samples	Degassed bath water	Degassed methanol
Ctrl and triplicates of pig vertebrae (G H_2O + G MeOH)		
Ctrl and triplicates of pig vertebrae (Dg H_2O + G MeOH)	*	
Ctrl and triplicates of pig vertebrae (Dg MeOH + GH_2O)		*
Ctrl and triplicates of pig vertebrae (DG H_2O + Dg MeOH)	*	*

2.9 Protein and Lipid Precipitation and SPE

After the solvent extraction was performed by one the three techniques, all samples were subject to protein and lipid precipitation and SPE before analysis by GC-MS or UPLC-DAD.

All dried samples were treated in the same manner. First PBS6 (0.1 M, pH 6, 3 mL) and ISTDs (50 and 250 ng of SB and DMI, respectively) were added. Based on the results from the section *2.5 Optimization of Protein and Lipid Precipitation*, 3 mL of ACN:MeOH (1:1, v/v, 3 mL) were added before samples were vortexed and stored at stored at -20 °C for 1 h to precipitate proteins and lipids. Samples were centrifuged (4000 RPM, 10 min) and supernatants were removed and evaporated to approximately 1 mL under a gentle steam of air at 70 °C. Samples were diluted to 4 mL with PBS6 and acidified with 200 µL of glacial acetic acid before loading. SPE was performed in the same manner as section *2.4 Optimization of SPE* where samples were extracted with CleanScreen CSDAU mixed-mode columns (200 mg, United Chemical Technologies, Bristol, PA). Columns were conditioned with methanol, distilled water and PBS6 (3 mL). Samples were then loaded by gravity and columns were sequentially washed with PBS6 (3 mL)

and acetic acid (0.1 M, 3 mL). Columns were dried under vacuum (10 in Hg, 5 min) before a final methanol wash (3 mL), where PB was eluted and collected. Columns were then dried under vacuum (15 in Hg, 10 min) and antidepressants were eluted using a mixture of ammonium hydroxide:isopropanol:EA (3:17:80, 2 x 3 mL). All extracts were evaporated to complete dryness under a gentle stream of air at 70 °C.

2.10 GC-MS Analysis of PB

The dried methanolic extracts were reconstituted in 100 µL ethyl acetate and 50 µL of a derivatizing agent (TMPAH, 50 µL) was added to perform flash methylation of the PB analytes before analysis by GC-MS. Extracts were transferred to the autosampler vials and analyzed on a PerkinElmer Clarus 600C GC-MS (PerkinElmer LAS, Shelton, CT) in the selected ion monitoring (SIM) mode, using electron impact ionization. A ZB-Drug-1 column (15 m x 0.25 mm x 0.25 µm, Phenomenex Inc., Torrance, CA) was used for separation and electron energy of 70 eV was used. Ions monitored were m/z 112, **169** and 184 (PB), and m/z 181, 195 and **196** (SB), where the ions in bold font were used for quantitative analysis. Aliquots (2 µL) were introduced into the injector which was maintained at 250 °C. The initial GC oven temperature was set to 60 °C and held for 3 min, before it was increased to 160 °C, at a rate of 100 °C /min. The temperature was then increased to 220 °C at a rate of 10 °C /min. Finally, the oven temperature ramp was set directly to 300 °C and held for 3 min. The total GC-MS run was approximately 20 min.

Positive analyte detection was based on two factors: retention time (within 3 standard deviation of standard samples) and peak area ratios of quantitative to qualifier ions (within 20 % of standard samples).

2.11 UPLC-DAD Analysis of DCIT, CIT, NTRIP and AMI

The dried SPE extracts were reconstituted in 500 µL of 0.1 % formic acid in 10:90 ACN:Ultra-pure water (A). Samples were centrifuged (13 000 RPM, 10 min) and supernatants were transferred to autosampler vials. Chromatographic separation was performed on an Acquity™ UPLC system (Waters Corp., Milford, MA) with a diode array detector DAD. A Kinetex C18 column (100 mm x 2.1 mm x 2.6 µm, Phenomenex, Torrance, CA) was used for analyte separation. The column temperature was held at 50 °C and separation was isocratic, using a mobile phase composition of 80:20 A:B (A: 0.1% formic acid in 9:1 acetonitrile:water; B: acetonitrile). The injection volume was 10 µL. The mobile phase flow rate was held constant at 0.300 mL/min for a total run time of 5 min. UV spectra were collected from 210-400 nm. Quantitative peak area comparisons were made using 240 nm for DCIT CIT, AMI and NTRIP, and 290 nm for DMI.

Positive analyte detection was based on two factors: retention time (within 3 standard deviation of standard samples) and UV spectrum (compared to spectrum of standard samples).

2.12 Statistical Analysis

The coefficient of variation (% CV) was used to measure reproducibility. Statistical analysis of the data was performed using StatPlus (2009 software, AnalystSoft Inc.). The mass-normalized response ratio (RR/m) for each extraction parameter studied was analyzed using two techniques; ANOVA (one-way) with Tukey's post-hoc analysis to assess differences between group means, and the validation criterion of ± 20 % mean variability to see if there is any overlap to confirm the statistical difference. A p value ≤ 0.05 was recognized as statistically significant.

Chapter 3

3 Results and Discussion

The main purpose of this work was to optimize and compare the performance of microwave-assisted extraction and ultrasonic solvent extraction methodologies to isolate drugs and metabolites from skeletal tissue in relation to a standard passive extraction. The effect of multiple variables, such as extraction solvent, sample mass, extraction time, solvent volume, microwave power and the effect of presence of gas in solutions undergoing ultrasonication on the extraction yield and rate were examined. Additionally, other sample preparation steps, such as the lipid and protein precipitation and SPE were also optimized.

3.1 Use of Animal Models

In the original research where this pig was used, pigs were selected due to their similarities to humans, especially in size and digestive and cardiovascular physiology [95]. Important parameters such as drug, dose, time between dose and death, and post-mortem environment can be controlled with animal models, unlike human models where drug history is usually unknown. As a result of the numerous physiological differences between humans and animals, it is important to note that animal studies should be used to develop analytical methods and to observe trends, but that levels obtained from animal models cannot be directly extrapolated to human cases.

3.2 Use of Skeletal Tissue in Forensic Toxicology

Although research laboratories have been looking at different techniques to isolate drugs and metabolites from skeletal tissue for the last decade, forensic toxicology laboratories still do not analyze bone for drugs in cases [11,12,13,14,22,47,93]. To help in the death investigation, this complex matrix would only be analyzed when conventional tissues, such as blood, urine and vitreous humor, are unavailable. The process of extracting the analytes from the solid matrix into solution can be challenging and time consuming.

3.3 Data Treatment

Drug recovery from bone cannot be accurately measured using methods standard to forensic toxicology laboratories. Here, data collected from GC-MS and UPLC-DAD are reported as mass-normalized response ratio (RR/m) rather than absolute concentration (e.g., ng/g). From the GC-MS, the response ratios of PB were calculated from the peak area of PB at a m/z of 169 relative to the peak area of SB at a m/z of 196. The RR of the antidepressants was determined by the peak area of a drug at 240 nm relative to the peak area of DMI at 290 nm. The value RR/m is proportional to analyte concentration, allowing for the comparison of relative drug levels obtained through different extraction methods and observing the influence of multiple experimental parameters on the recovery. Data are reported this way because the determination of absolute drug concentration in bone is complex since drug recovery from bone is indeterminable, given that bone cannot be spiked homogeneously, and there are no standardized methods of sample preparation for this tissue type. Concentration measurements may therefore

be misleading, since reported drug concentration from a given bone may vary between laboratories as isolation techniques may differ from one another (i.e. ground versus bone slivers) [2,77]. Other than the extraction method, the recovered drug level can vary depending on the anatomical location of the bone (ex: central versus peripheral bones) and even within a bone (ex: epiphyseal versus diaphyseal) [11,12,13,96]. Given that the purpose of this work was to compare the performance of different extraction methods, the RR/m parameter was selected as an appropriate measure to compare analytical responses of different samples.

3.4 Optimization of Solid Phase Extraction (SPE)

Optimization of the solid-phase extraction method was completed by examining the effect of SPE column and hexane wash on the extraction yield.

3.4.1 SPE Column Comparison

Three different solid-phase columns were compared using samples containing 100 ng/mL of all analytes. Figure 8 demonstrates no significant difference in yield between SPE columns. The UCT Clean screen column was chosen based on its lower cost.

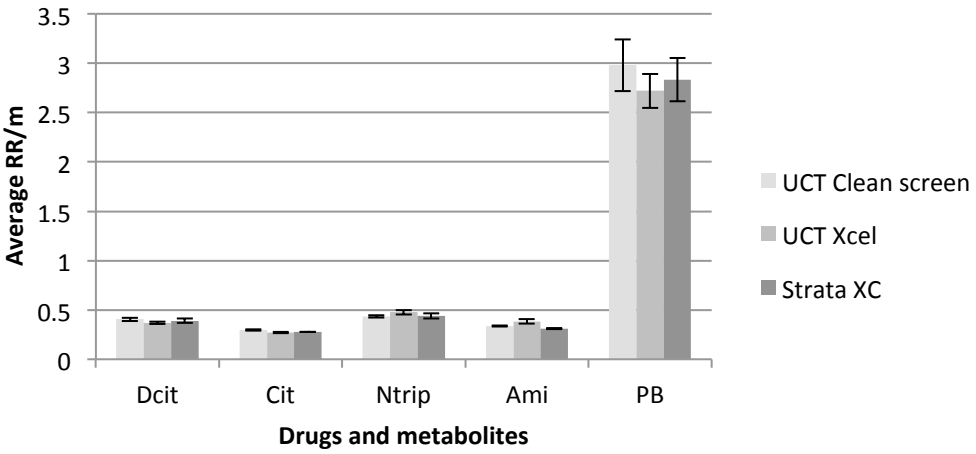


Figure 8: Comparison of solid-phase extraction column

3.4.2 Effect of Hexane Wash

Based on the results of the prior evaluation, a second set of extractions was performed to examine the influence of a hexane wash step during the SPE on the extraction yield. At first view, the results showed a major decrease in background noise, which seemed promising. Upon further examination, it seemed the analyte signals had also decreased along with background noise. By comparing the peak areas (indicated below the retention time in figure 9), the loss of analytes with the hexane wash is clear.

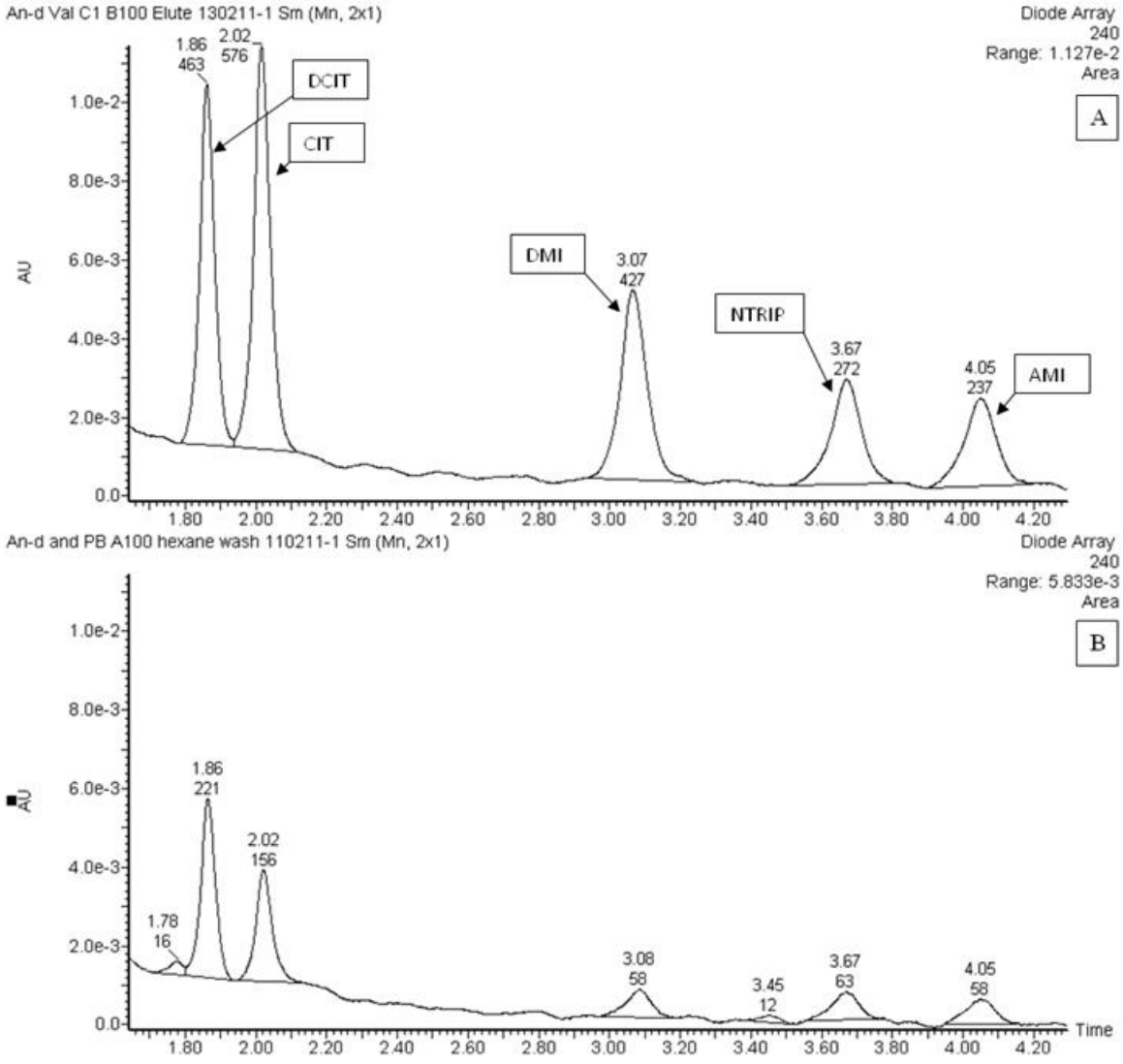


Figure 9: UPLC-DAD of DCIT, CIT, NTRIP, and AMI (250 ng of DMI as ISTD) without a hexane wash (A) and with hexane wash (B)

3.5 Protein and Lipid Precipitation

Optimization of the lipid and protein precipitation method was carried out by examining the effect of solvent and time period stored at -20 °C on the protein and lipid precipitation step.

3.5.1 Effect of Solvent

Four different precipitation solutions were examined to optimize the extraction yield, ACN:MeOH (1:1 v/v), ACN, acetone:MeOH (1:1 v/v) and acetone. Samples (n = 3) were made of 1 mL BTE spiked with 100 ng/mL of all analytes, where no significant differences were observed for all precipitation solutions (figure 10). The ACN:MeOH mixture was chosen for consistency purposes since prior methodology, such as the validation, was performed with this mixture. Due to lower cost of acetone, the acetone or acetone:MeOH solutions should be considered as a precipitation mixture in future research.

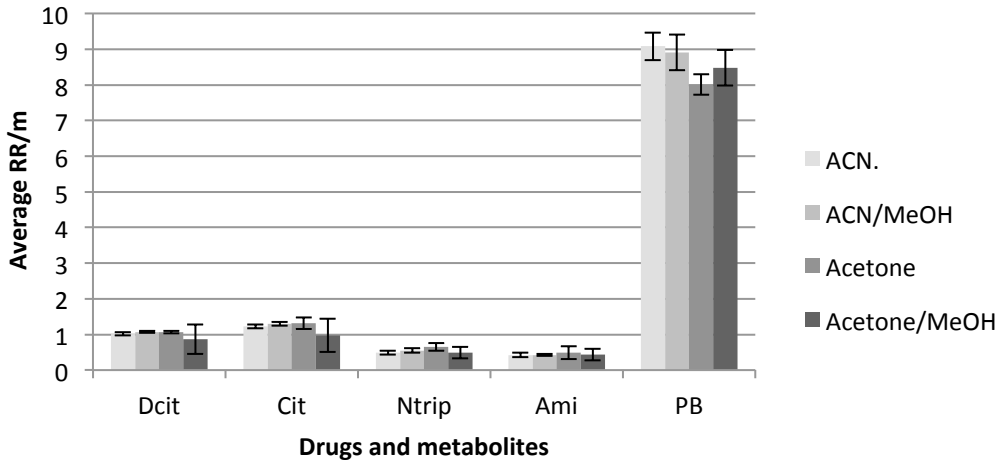


Figure 10: Comparison of solvent for protein and lipid precipitation

3.5.2 Effect of Precipitation Time

Furthermore, the lipid and protein precipitation step was optimized by observing the influence of storage time at -20 °C to precipitate proteins and lipids on the extraction yield of sample at low and high analyte concentrations. Samples (1 mL) containing 50 or 1000 ng/mL of PB, DCIT and

CIT and 100 or 2500 ng/mL of NTRIP and AMI were subject to 1 or 24 h of storage. No significant difference was calculated between the set of samples (figure 11).

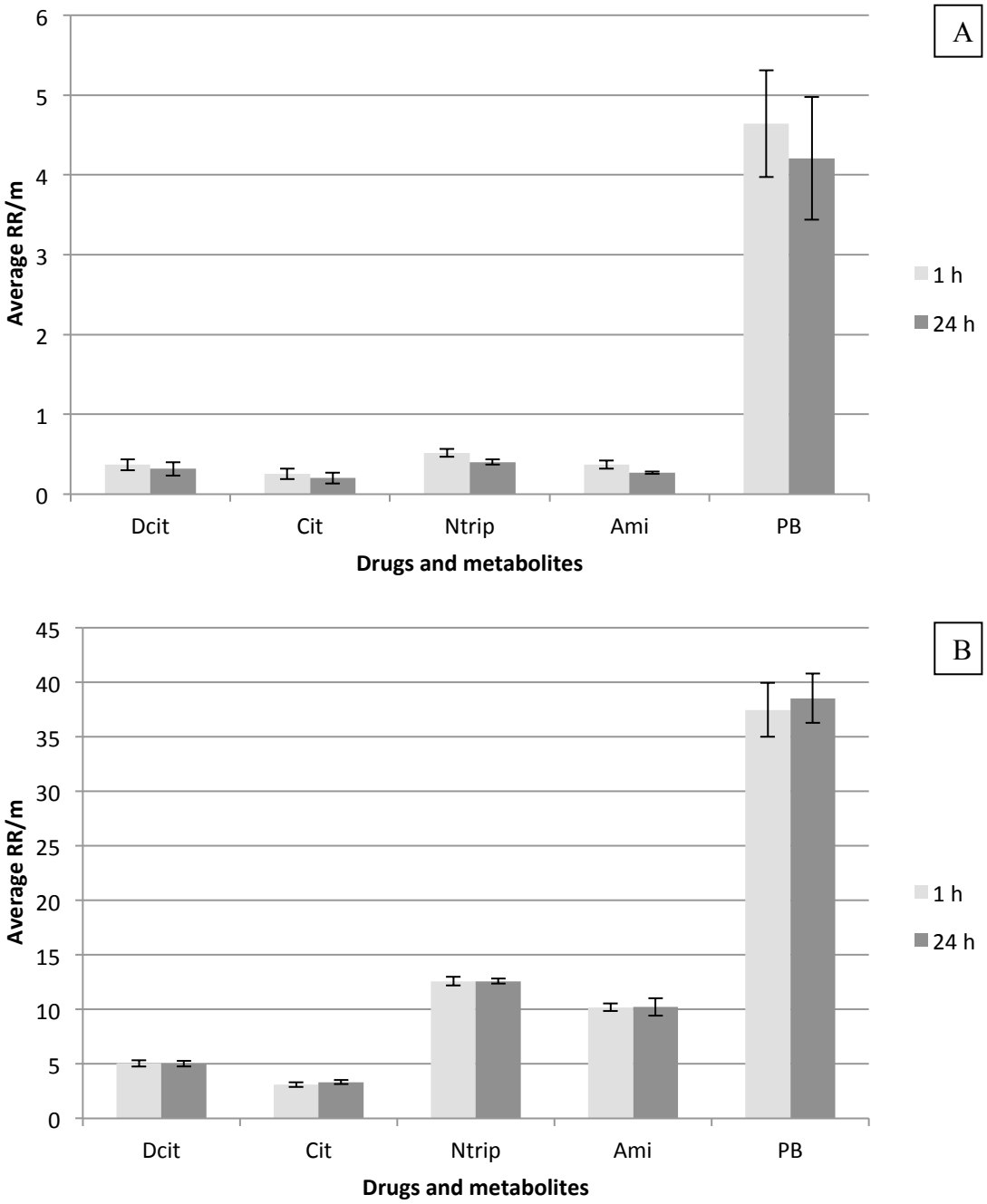


Figure: 11: Comparison of storage time for lipid and protein precipitation of (A) 50 and 100 ng/mL, and (B) 1000 and 2500 ng/mL of DCIT, CIT and PB, and NTRIP and AMI, respectively

3.6 SPE Method Validation

The precision of the method was measured as the percent coefficient of variation of RR with 6-point standard curves, where replicates (n = 8) were made of spiked BTE. With the exception of PB, which reached 31.9 %, all % CV of interday replicates were below 30 %, varying from 0.2 to 22.8 % for DCIT, 8.3 to 15.1 % for CIT, 1.9 to 11.9 % for NTRIP, 0.2 to 15.5 % for AMI and 5.0 to 31.9 % for PB. Replicates with a % CV higher than 20 % were observed at concentrations lower than 25 ng/mL. For the intraday replicates, a maximum % CV of 12.7, 9.8, 8.8, 26.8 and 8.6 % was calculated for DCIT, CIT, NTRIP, AMI and PB, respectively. A standard curve was run with each analysis to measure the % CV. These curves confirmed the variability did not exceed the validation criterion of 20 % and that the cut off level was no different from the previous value obtained. Linearity of RR was observed between 5 and 2000 ng/mL for DCIT, CIT and PB 5 and 5000 ng/mL for NTRIP and AMI. R^2 values were no lower than 0.996, 0.996, 0.980, 0.997 and 0.997 for DCIT, CIT, NTRIP, AMI and PB, respectively. Figures 12 and 13 demonstrate standard curves for PB, DCIT and CIT, and NTRIP and AMI, respectively.

The cut off level and LOQ were determined with replicates (n = 9) of 5, 10 and 25 ng/mL made from drug free BTE. The cut off level for all analytes was 5 ng/mL, while the LOQ was 10 ng/mL for DCIT, CIT and PB, and 25 ng/mL for NTRIP and AMI.

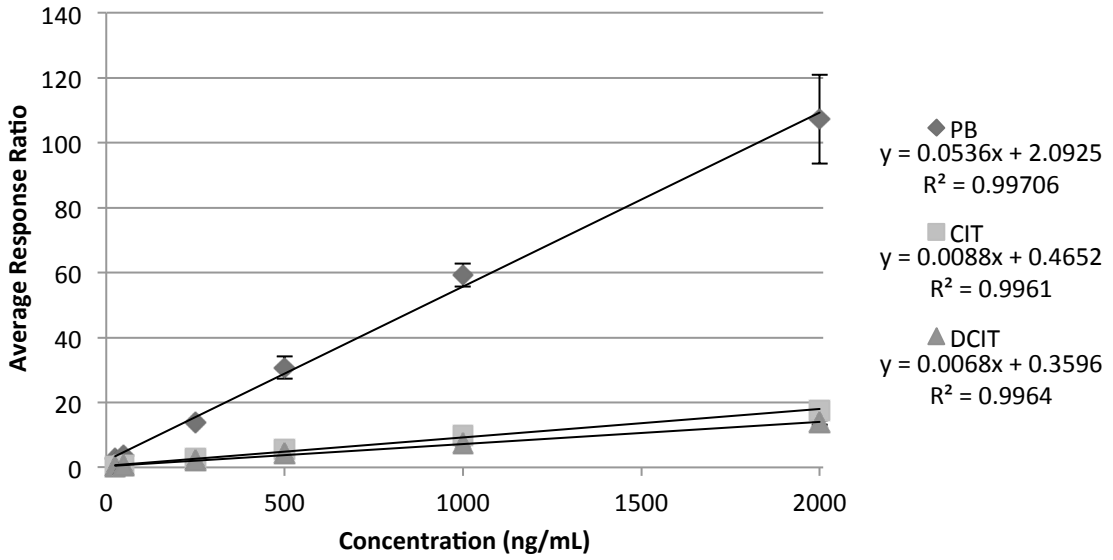


Figure 12: Average response ratio of PB, DCIT and CIT in function of concentration

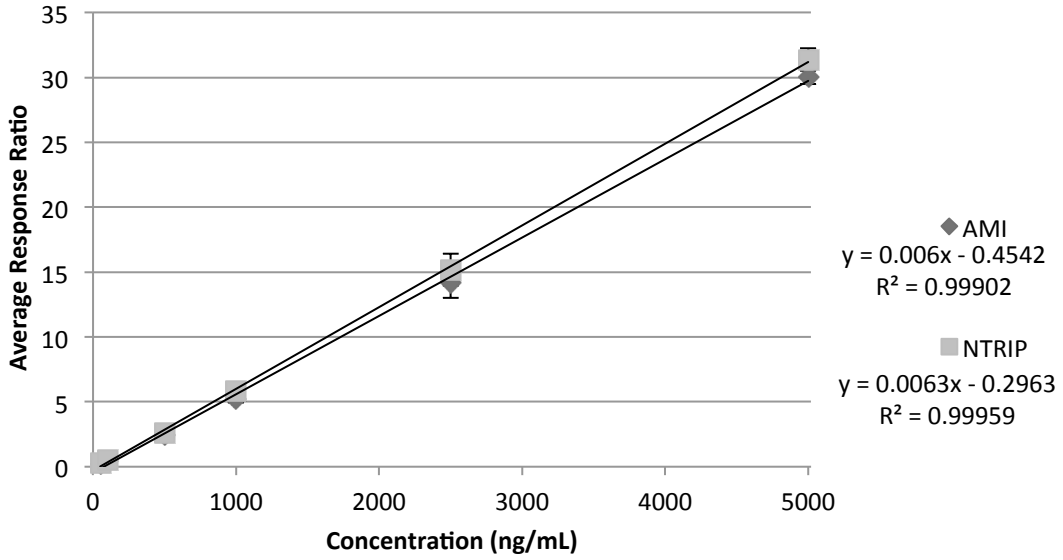


Figure 13: Average response ration of NTRIP and AMI in function of concentration

3.7 Stability

It is important to study the stability of drugs and metabolites under all extraction conditions. Extraction methods such as MAE and USE are relatively new to forensic toxicology laboratories

and have not been studied as intensively as the passive incubation technique. Furthermore, compared to the standard conditions of passive extraction, microwave irradiation reaches high temperatures more quickly and ultrasonic agitation creates cavities of extremely high pressure and temperature. These aggressive conditions could lead to analyte degradation by hydrolytic losses, limiting their use in practice.

Drug stability at two different concentrations, low-mid and mid-high, were examined in MeOH and EA for passive, microwave and ultrasonic extraction. Control bone (2 g) was spiked with 200 or 2000 ng/mL of DCIT, CIT and PB, and 500 or 5000 ng/mL of NTRIP and AMI, and solvent volume was brought up to 10 mL. The stability of each drug was determined by comparing analyte RR after being exposed to a certain extraction method for a pre-determined time periods. ANOVA (one way) with post-hoc analysis, along with the validation criterion of ± 20 % mean variability, were used to determine if the variation in RR values between different extraction times was significantly greater than that observed between replicates at each extraction time. In general, no significant effect was observed from time of exposure on assay response for all three extraction methods.

3.7.1 Standard Passive Extraction Stability

Overall the stability study showed no significant loss of any analyte throughout 96 h of passive solvent extraction. Figure 14 shows an example of the average response ratio of (A) 200 ng/mL and (B) 2000 ng/mL of pentobarbital in function of standard passive extraction time in MEOH and EA.

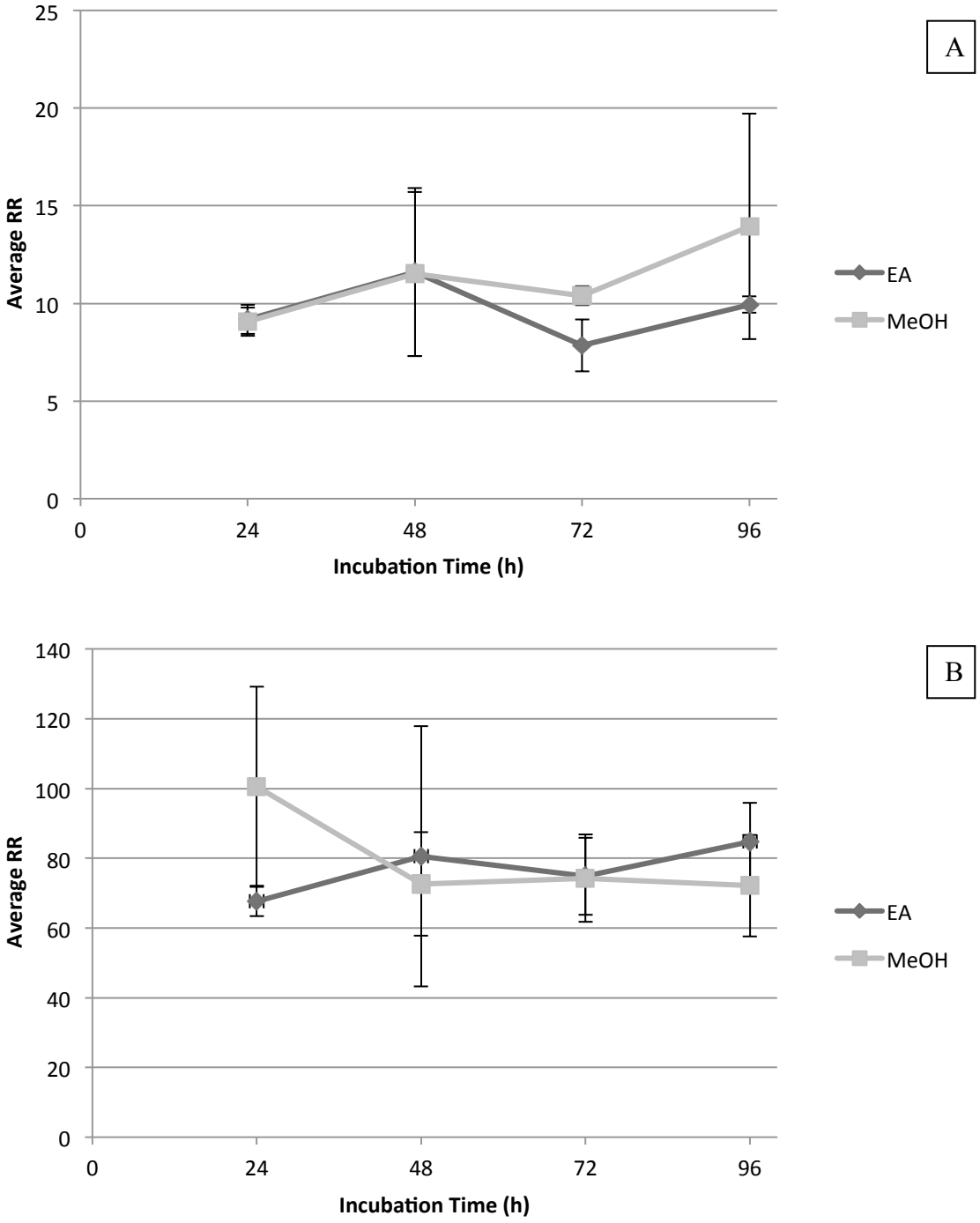


Figure 14: Stability of PB, (A) 200 ng/mL and (B) 2000 ng/mL, in MeOH and EA through 96 h of passive extraction

3.7.2 Microwave-Assisted Extraction Stability

The drugs were stable through 30 min of microwave irradiation, and no significant analyte loss was noticed. A trend at 30 min of irradiation was observed, where the average RR increased, most likely due to solvent evaporation. An example is illustrated in figure 15.

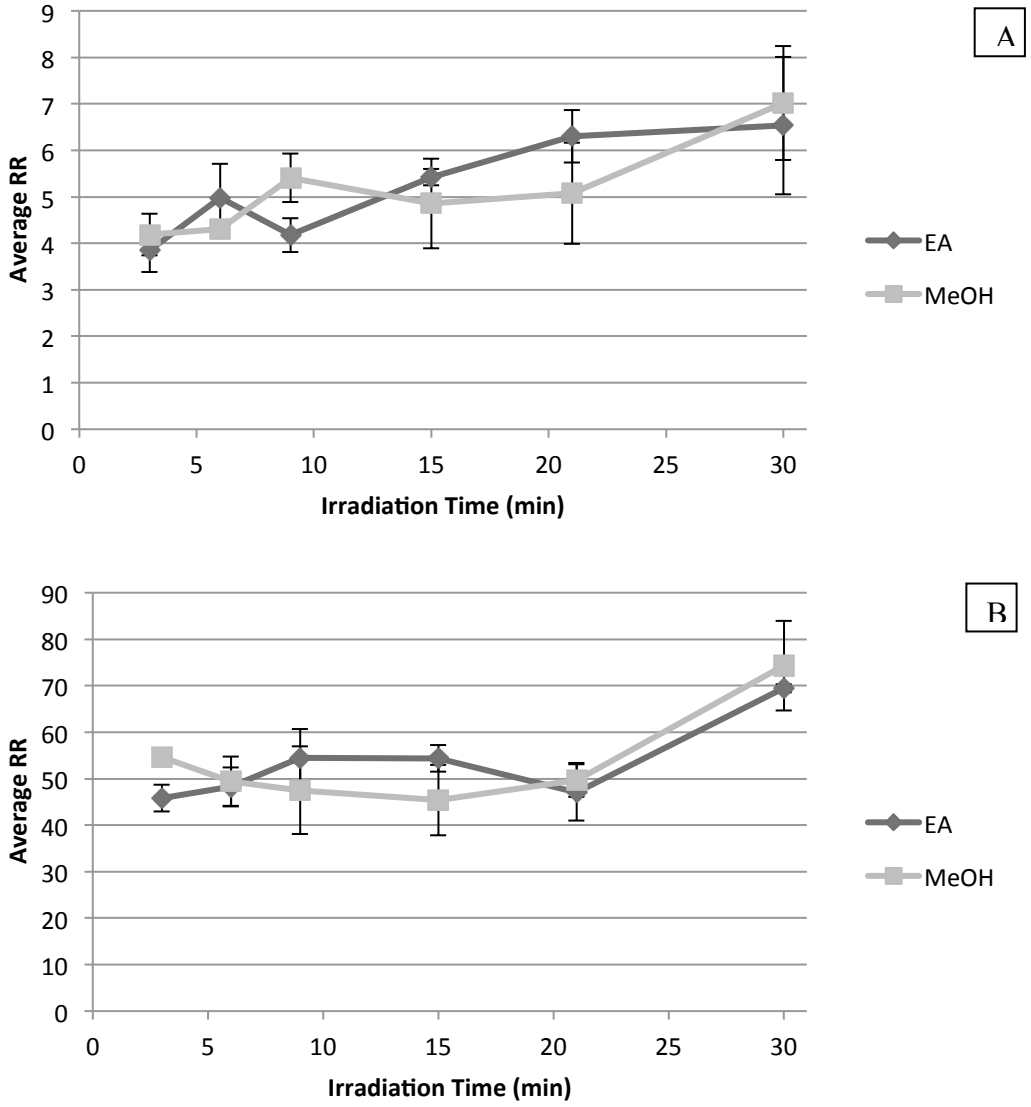


Figure 15: Stability of CIT, (A) 200 ng/mL and (B) 2000 ng/mL, in MeOH and EA through 30 min of microwave irradiation

3.7.3 Ultrasound Solvent Extraction Stability

A slight decrease in average RR was observed throughout ultrasonication time as illustrated in figure 16. No significant difference was observed, with the exception of NTRIP at 500 ng/mL in EA.

A decrease in RR reported does not necessarily mean a loss of the analyte due to instability. Other factors contribute to observing a decrease in levels, including the possibility of drugs being adsorbed onto the glass or bone and the increase in background material extracted with time. In addition, as the ultrasonication continues over time, the amount of interfering components desorbed from bone, along with the analytes, accumulate into the solvent, possibly increasing the baseline and appearing as a decrease in analyte level. No clear trend was observed, given that the losses are not consistent at both concentrations and in both solvents. Therefore, the nature in changes in assay response of this extraction method was undetermined. It is therefore not clear if the loss of analytical signal represents drug degradation or suggests another process such as adsorptive loss.

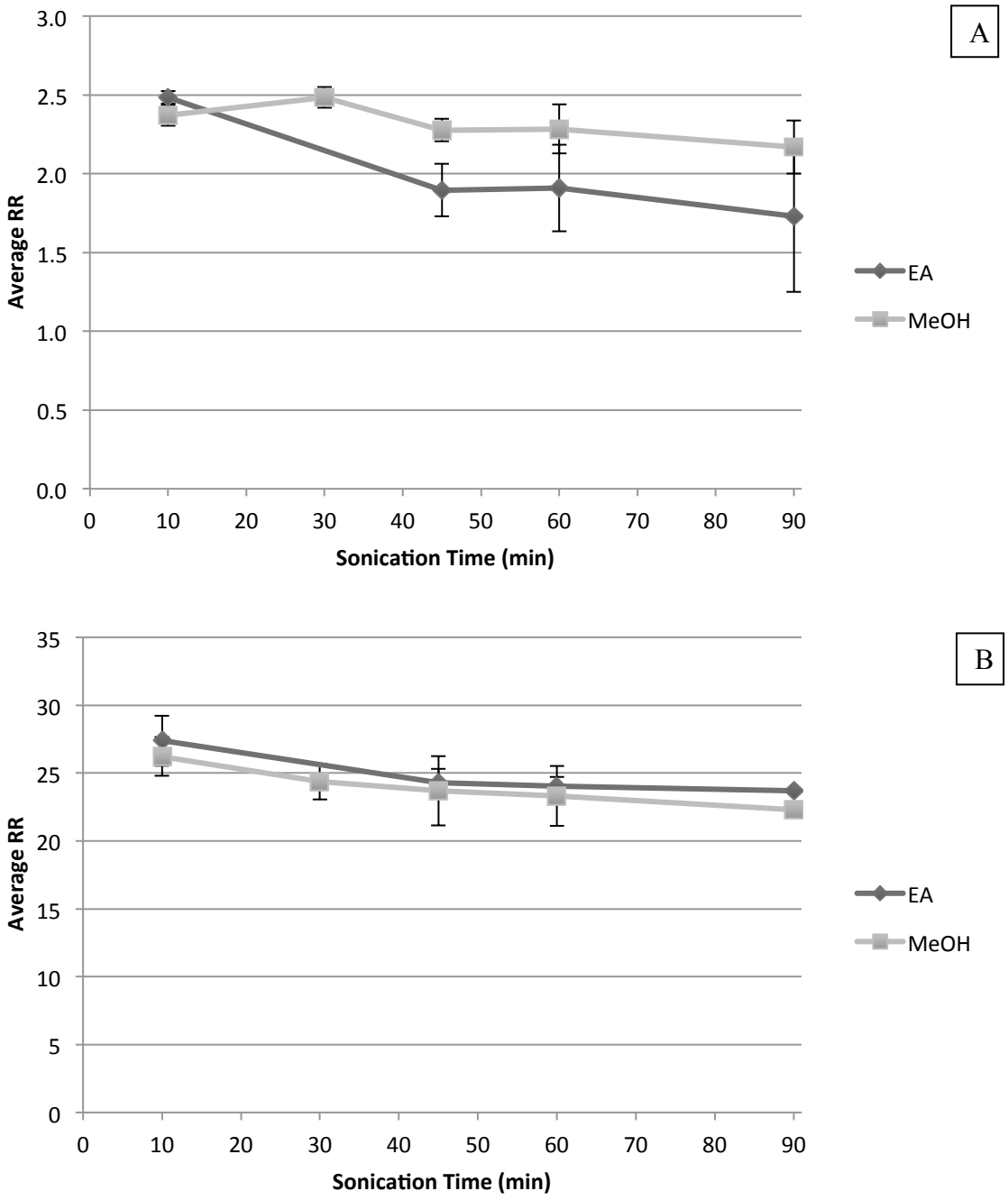


Figure 16: Stability of AMI, (A) 500 ng/mL and (B) 5000 ng/mL, in MeOH and EA through 90 min of ultrasonic agitation

3.8 Optimization/Characterization of Drug Extraction from Bone

Optimization of the extraction methods was carried out by sequential extractions of skeletal tissue in aliquots of fresh solvent. After certain predetermined periods of time, the solvent was recovered and replaced with a fresh solvent. Each solvent fraction recovered was analyzed separately.

3.8.1 Effect of Extracting Solvent

In general, samples extracted in MeOH tended to have a significantly greater yield compared to those extracted in EA. This trend was observed in all three extraction methods, with one exception.

3.8.1.1 Standard Passive Extraction

Statistical analysis showed a significant difference ($p \leq 0.05$) between analyte recovery in long term passive incubation samples in MeOH and those incubated in EA for PB and NTRIP for both masses, and DCIT, CIT and AMI for 2 g samples only (figure 17 A).

As for short term interval passive incubation, a significant difference was observed for CIT, NTRIP, AMI and PB for both masses, and DCIT for 2 g samples (figure 17 B).

3.8.1.2 MAE

All samples submitted to microwave irradiation showed significant differences when comparing extraction yields of samples extracted in MeOH and those extracted in EA in both masses for all analytes (figure 17 C).

3.8.1.3 USE

A statistical difference in extraction yield was observed for the majority of the samples which underwent ultrasonication, where samples extracted in MeOH resulted in higher analyte recovery compared to sample extracted in EA, with one exception. DCIT was not detected in all samples subjected to USE in EA, and was only slightly above the detection limit in the first round of samples extracted in MeOH (figure 17 D). Surprisingly, CIT levels were significantly higher when extracted in EA compared to those extracted in MeOH for the 1 g samples. This was the only occurrence where EA surpassed MeOH as extracting solvent. Levels were significantly higher in MeOH in both masses for NTRIP and AMI, and at the 2 g samples for PB.

3.8.2 Effect of Sample Mass

The effect of sample mass did not appear to influence the mass-normalized extraction yield, and no common trend was observed in all extraction methods or extraction solvents, with one exception (figure 17). Pentobarbital levels were significantly higher in the 1 g samples compared to the 2 g samples submitted to microwave irradiation in methanol. No significant differences were observed with any other analyte when comparing different mass in both solvents and in all

three extraction methods, suggesting that the increase in sample mass resulted in proportional absolute analyte yield. Therefore, the masses sampled were not too large for the solvent volume.

3.8.3 Effect of Extracting Solvent and Sample Mass on Extraction Time

3.8.3.1 Standard Passive Extraction

The effect of extraction solvent and sample mass on the extraction rate was also examined. It appears the majority of the analytes were extracted within 24 and 48-72 h of standard passive extraction in MeOH and EA, respectively. Exceptions were observed where levels of NTRIP and AMI from the 2 g samples extracted in EA were still near the cut off level even after 96 h of passive incubation. On the other hand, the sample mass did not seem to have any effect on the extraction rate for samples incubated passively. Figure 18 demonstrates an example of levels of AMI recovered from long interval passive solvent extraction of 1 and 2 g of porcine bone in 10 mL of (A) EA and (B) MeOH.

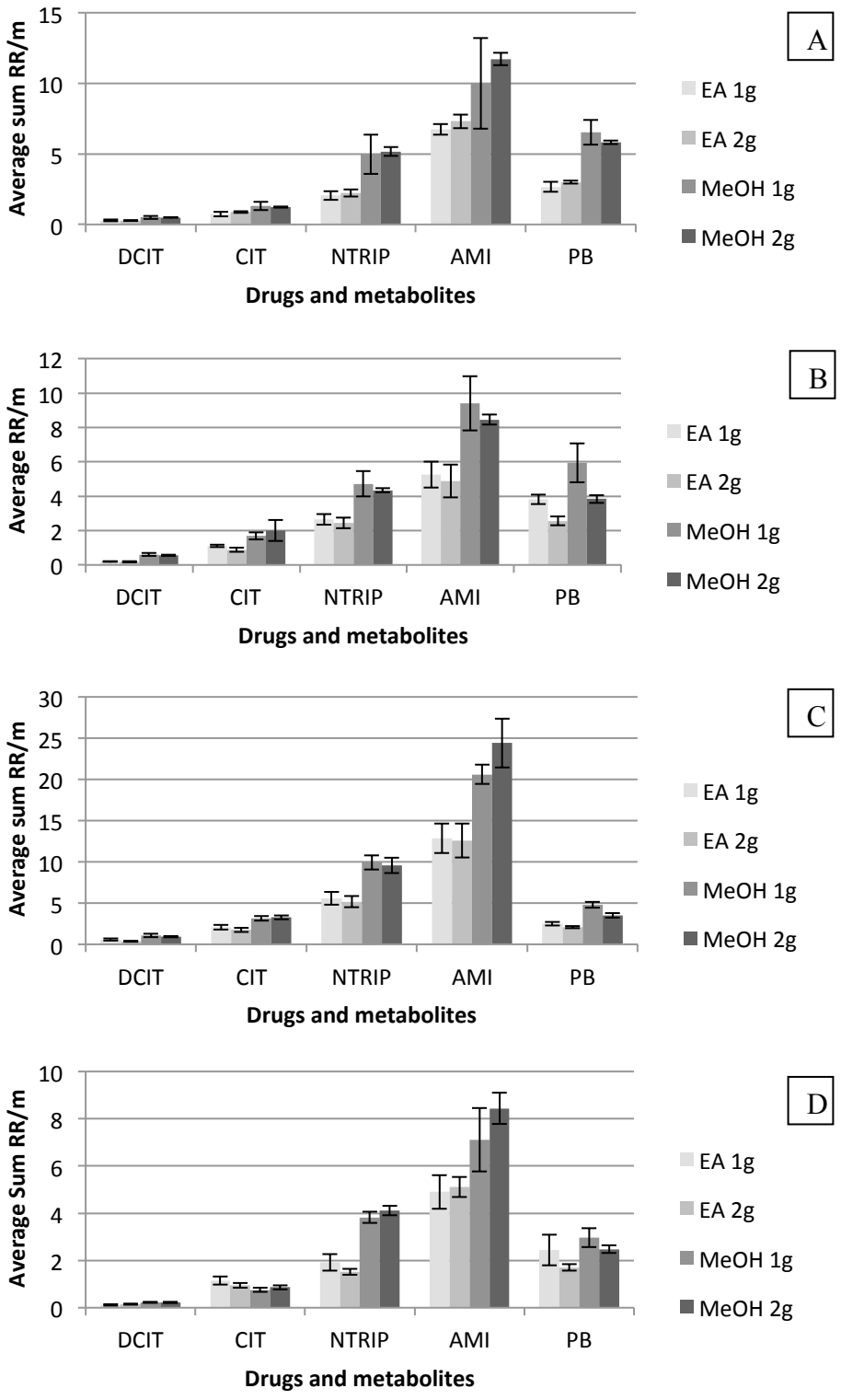


Figure 17: Average (n = 3) of the sum of RR/m of sequential extraction aliquots of DCIT, CIT, NTRIP, AMI and PB from 1 and 2 g samples extracted in MeOH and EA (A) Long and (B) short term standard passive extraction, (C) MAE and (D) USE

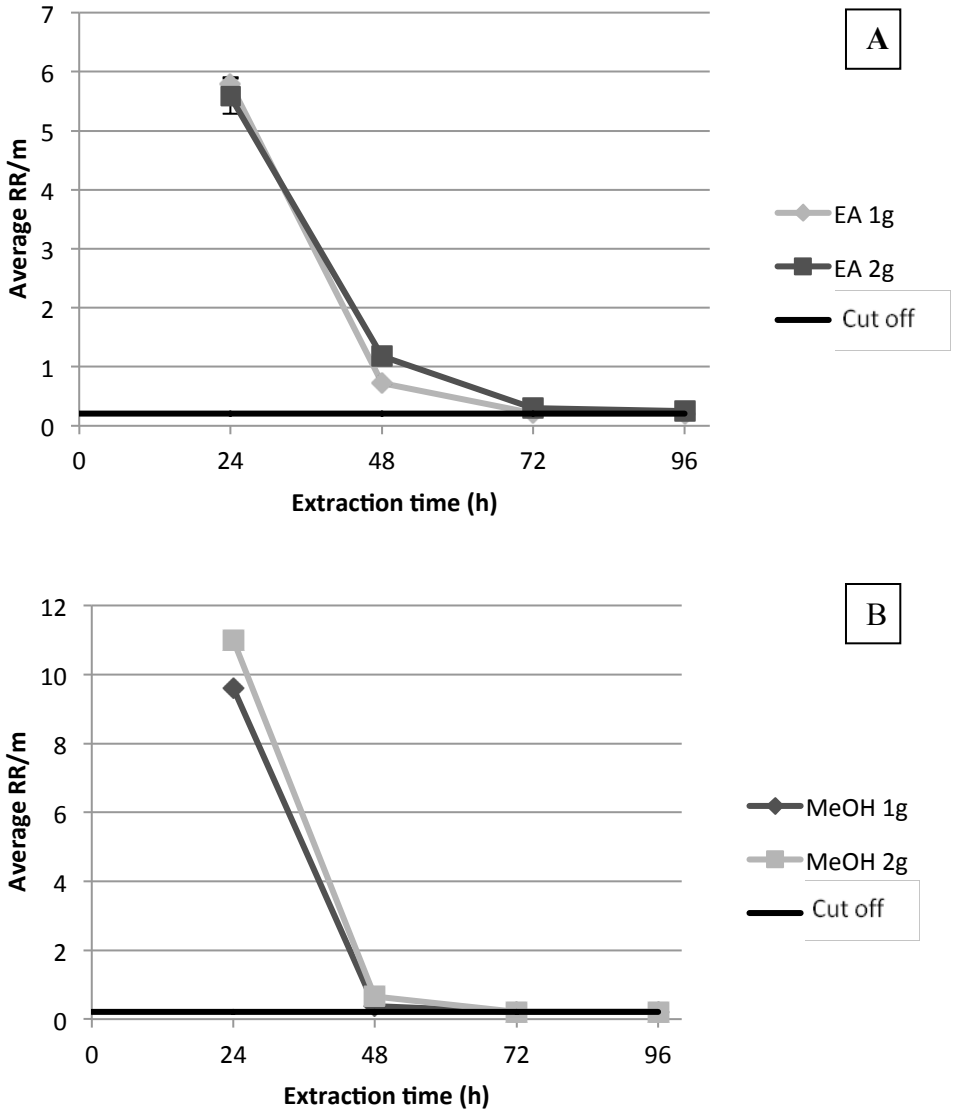


Figure 18: Average RR/m of AMI from 1 and 2 g bone samples submitted to long term interval standard passive extraction in 10 mL of (A) EA and (B) MeOH, in function of passive incubation time

Upon review of the evaluation of the long term intervals of passive incubation, results demonstrated maximum yield within the first 24 h in MeOH. For this reason, a second set of samples was incubated to establish the actual extraction time for maximum yield by examining

the incremental recovery with short term sampling. Shorter intervals of passive incubation were not initially considered since extraction times reported in the literature have not been shorter than 12 h, with one exception. Desrosiers et *al.* have examined samples submitted to passive extraction for periods of time including 1, 6 and 12 hours, where the majority of the analytes had reached maximum yield within 6 h [14]. The results seemed to be drug dependent.

Results from the short intervals of passive incubation indicate that maximum recovery of DCIT, CIT, NTRIP, AMI and PB was achieved within 6, 21, 15, 10 and 30 min, respectively, in both extracting solvents. In this short time, no major difference was observed when comparing the extraction time of samples extracted in MeOH and those extracted in EA. It is important to note that no report was found in the literature regarding short term passive incubation with fresh bone. As noted before, porosity of bone increases with time, thus fresh bone may behave differently. Levels of nortriptyline from short intervals of passive solvent extraction are illustrated in figure 19. No trends were observed on the extraction rate with respect to the sample mass.

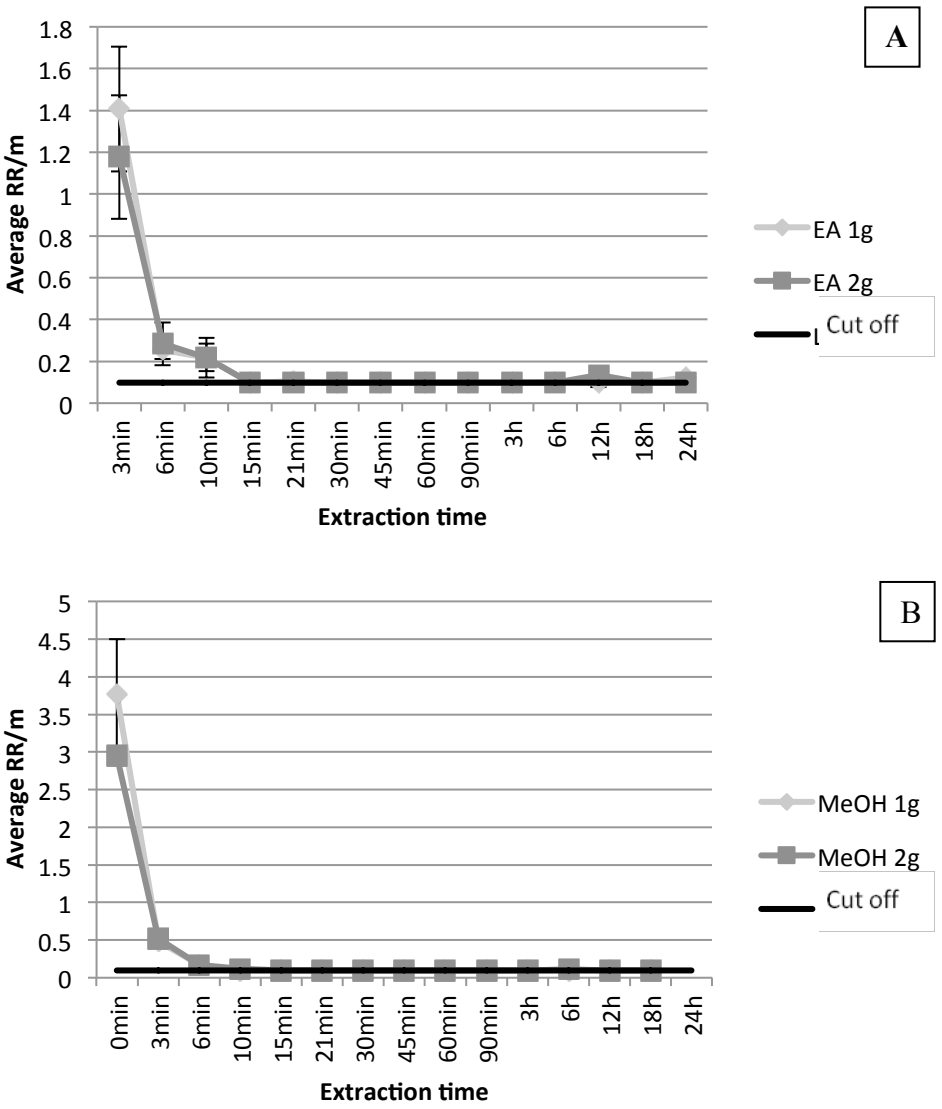


Figure 19: Average RR/m of NTRIP from 1 and 2 g bone samples submitted to short term interval standard passive extraction in 10 mL of (A) EA and (B) MeOH, in function of passive incubation time

3.8.3.2 MAE

Similarly to passive solvent extraction, the MAE rate of extraction seemed faster with MeOH than EA (see figure 20) while the sample mass did not have any effect on the speed of analyte recovery. After 6 minutes of irradiation there was no further detectable recovery for DCIT, while

all other analytes levels were near the cut off level up to 9 and 15 min for samples extracted in MeOH and EA, respectively. The average mass-normalized response ratio of NTRIP of 1 and 2 g bone samples submitted to microwave irradiation is illustrated in figure 20.

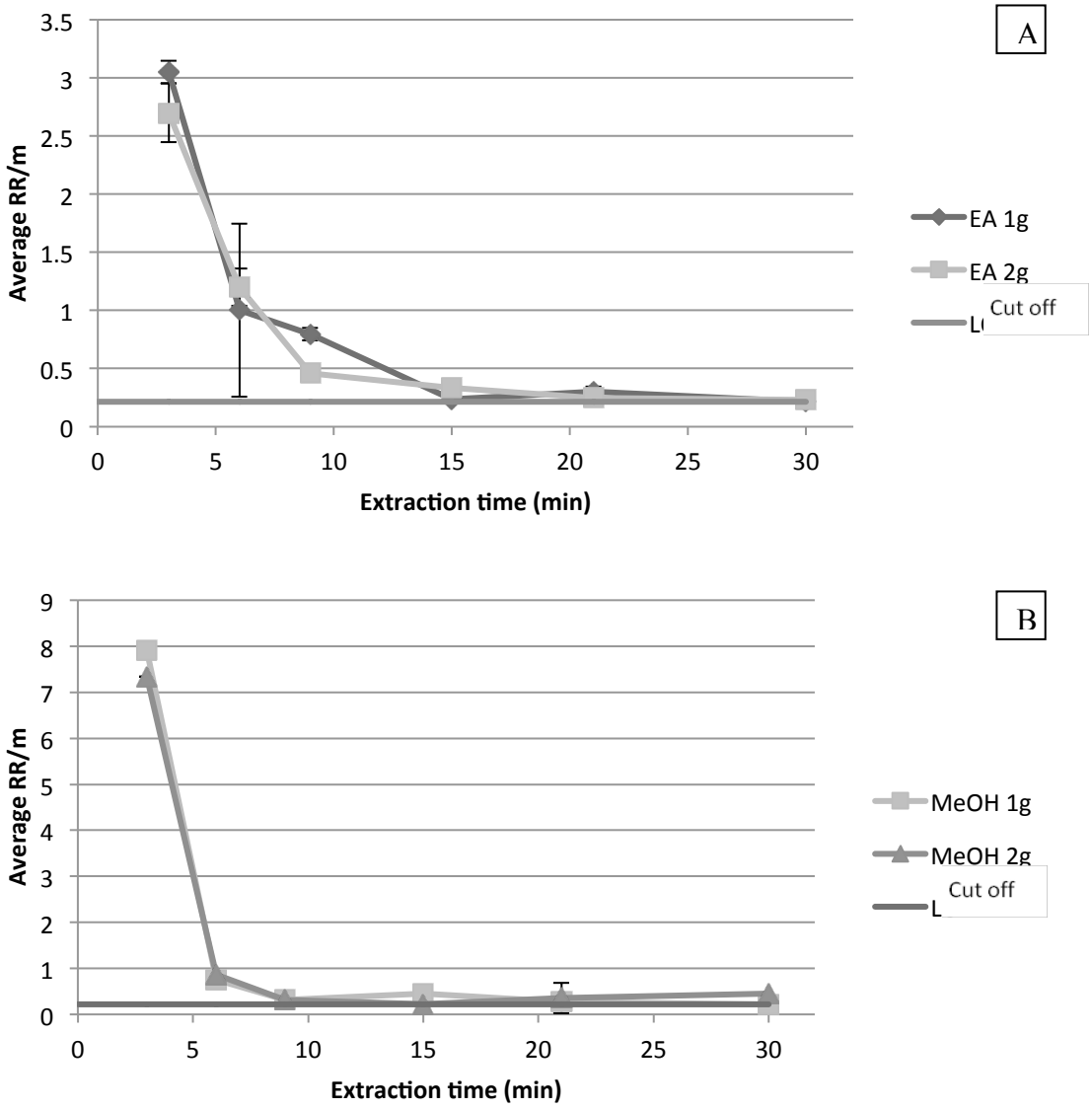


Figure 20: Average response (RR/m) of NTRIP from 1 and 2 g bone samples submitted to MAE in 10 mL of (A) EA and (B) MeOH, in function of irradiation time.

3.8.3.3 USE

The majority of the analytes recovered from samples extracted in MeOH achieved maximum yield within 30-45 min of ultrasonic agitation, while it took 45-60 min for samples extracted in EA. Some exceptions occurred, where even after 90 min of ultrasonic agitation in EA levels of CIT and AMI for 1 g and both masses, respectively, were still above the cut off level. Similar results were observed with standard passive extraction. The average mass-normalized response ratio of CIT of 1 and 2 g bone samples submitted to ultrasonic agitation in (A) EA and (B) MeOH is illustrated in figure 21.

3.8.4 Effect of Solvent Volume

Based on the assessment of the influence of extraction solvent, sample mass and extraction time, the influence of the solvent volume on the extraction yield was investigated in a second set of extractions. Since MeOH resulted in greater rate and recovery, it was chosen over EA for all further extractions. Also, since the sample mass did not have any effect on the recovery and due to the limited amount of bone, all samples were prepared with 1 g of ground bone. Thus samples (n = 3) containing 1 g of bone were extracted in 5, 10 or 15 mL of MeOH. Furthermore, the extraction times were cut down to 72 h, 15 min and 45 min for passive, MAE and USE, respectively.

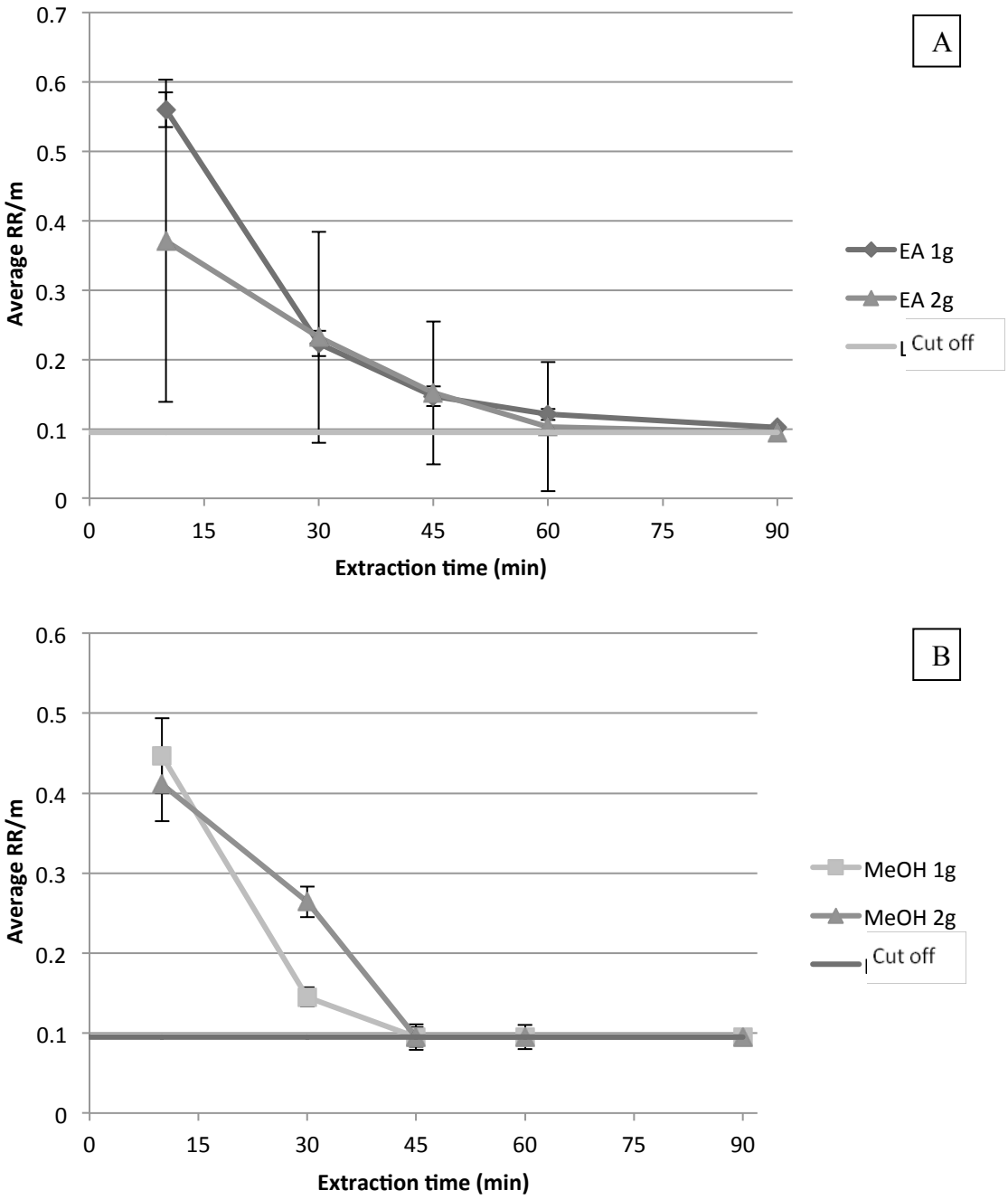


Figure 21: Average RR/m of CIT from 1 and 2 g bone samples submitted to USE in 10 mL of (A) EA and (B) MeOH, in function of ultrasonic agitation time.

No significant differences were observed for all analytes passively incubated in different MeOH volumes, with the exception of PB. The 15 mL samples yield three times the level of drugs extracted in the 5 and 10 mL samples (figure 22 A). For samples irradiated by microwaves, similarly to the passive incubation samples, ANOVA one-way results show no significant difference in all analytes (figure 22 B). Finally, for samples submitted to USE, most analytes showed no significant difference, but lower solvent volumes (5 and 10 mL) resulted in significantly higher extraction yields than 15 mL samples for DCIT and CIT.

3.8.5 Effect of Microwave Power

The influence of microwave power on the extraction yield was investigated using samples (n = 3) containing 1 g of porcine bone in 5 mL of MeOH. Samples were irradiated for a total of 15 min at a power of 100, 80, 50 or 20 %, which are equivalent to 1100, 880, 550 and 220 W, respectively. With the exception of AMI and NTRIP, no significant difference was observed when comparing analyte yields from samples irradiated at different powers. A significantly higher yield resulted from samples extracted at 100 % than those extracted at 80, 50 and 20 % for AMI and NTRIP (figure 23).

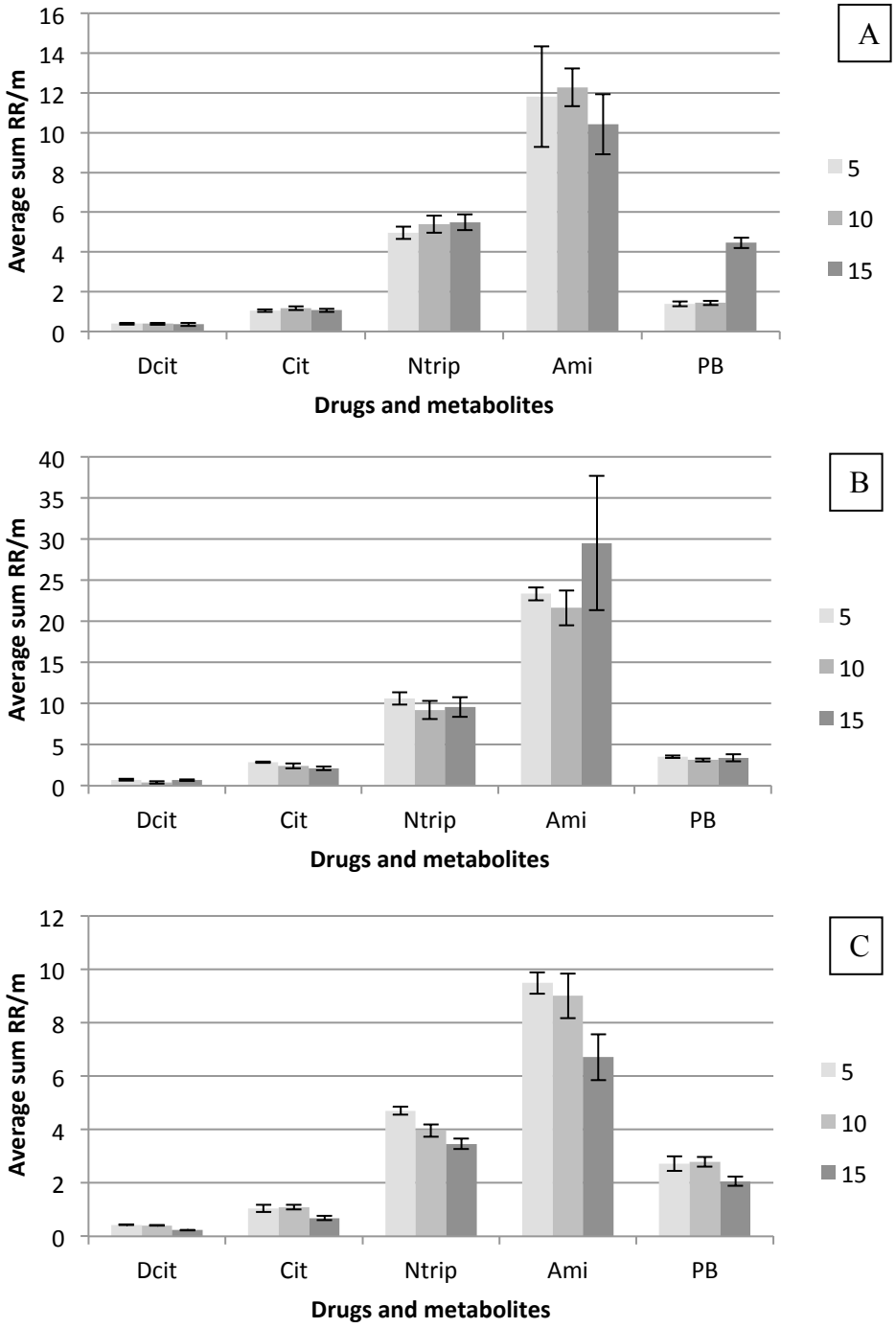


Figure 22: Average (n = 3) of the sum of RR/m of sequential extraction aliquots of DCIT, CIT, NTRIP, AMI and PB from 1 g bone samples extracted in 5, 10 and 15 mL of MeOH by (A) standard passive extraction, (B) MAE and (C) USE

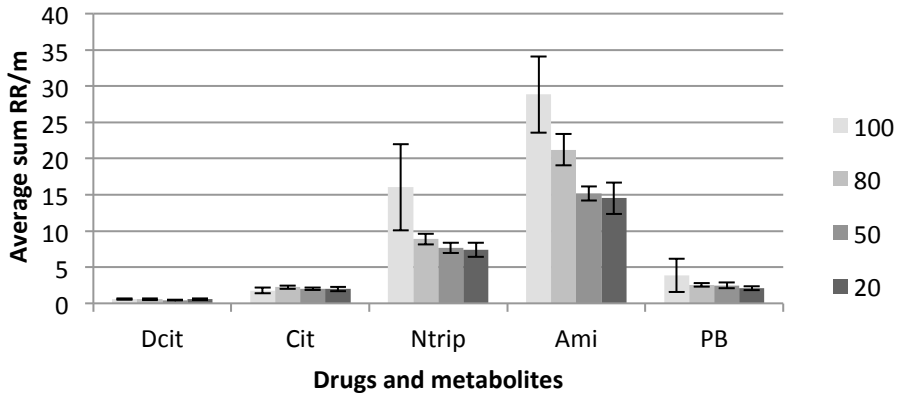


Figure 23: Average (n = 3) of the sum of RR/m of sequential extraction aliquots of DCIT, CIT, NTRIP, AMI and PB from 1 g bone samples submitted to MAE at 100, 80, 50 and 20 % power in 5 mL of methanol

3.8.6 Effect of Presence of Dissolved Gases in Bath Water and Extraction Solvent in USE

The influence of dissolved gases in bath water and extraction solvent on the extraction yield of samples submitted to ultrasonic agitation was examined. Samples containing 1 g of porcine bone in 5 mL of MeOH were used and vacuum degassing of water (Dg H_2O) and methanol (Dg MeOH) was performed prior to the extraction. As observed in figure 24, ANOVA (one-way) showed significant difference for AMI between degassed bath water with degassed extraction solvent samples (Dg H_2O + Dg MeOH) and all other samples submitted to ultrasonic waves. All other analytes did not show any significant difference in yields when comparing the different extraction conditions.

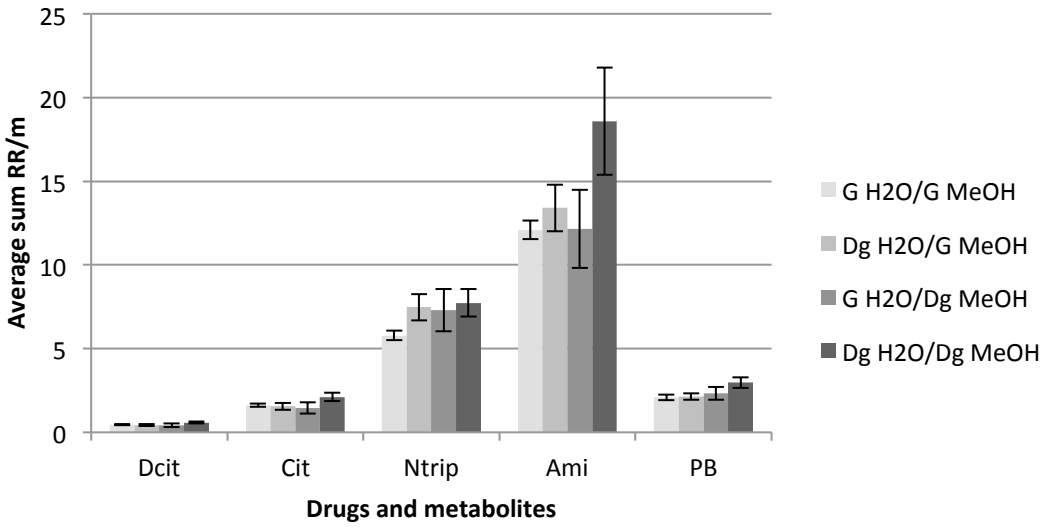


Figure 24: Average (n = 3) of the sum of RR/m of sequential extraction aliquots of DCIT, CIT, NTRIP, AMI and PB from 1 g bone samples submitted to USE in 5 mL of MeOH with degassed bath water and extraction solvent

When comparing the recovery levels of the three extraction methods, higher yields were obtained in general when samples were being irradiated by microwaves compared to passively incubated or submitted to ultrasonic waves (figure 22).

3.9 PB Analysis by GC-MS

Pentobarbital in porcine bone was analyzed by GC-MS and had a retention time of approximately 6.80 min (figure 25). Chromatograms of negative controls show minimum background noise at these retention times, causing little to no interference with the analyte and ISTD signals. Pentobarbital and secobarbital were identified with a combination of retention time and peak ratios of quantitative to qualitative ions (figure 26 (A) PB and (B) SB).

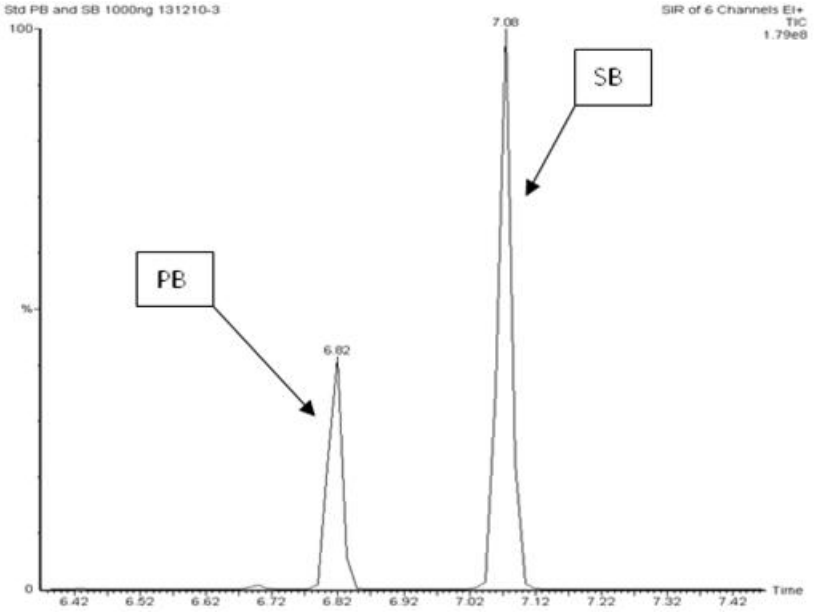


Figure 25: Total ion chromatogram (TIC) of 1000 ng of PB (6.82 min) and SB (7.08 min) standards

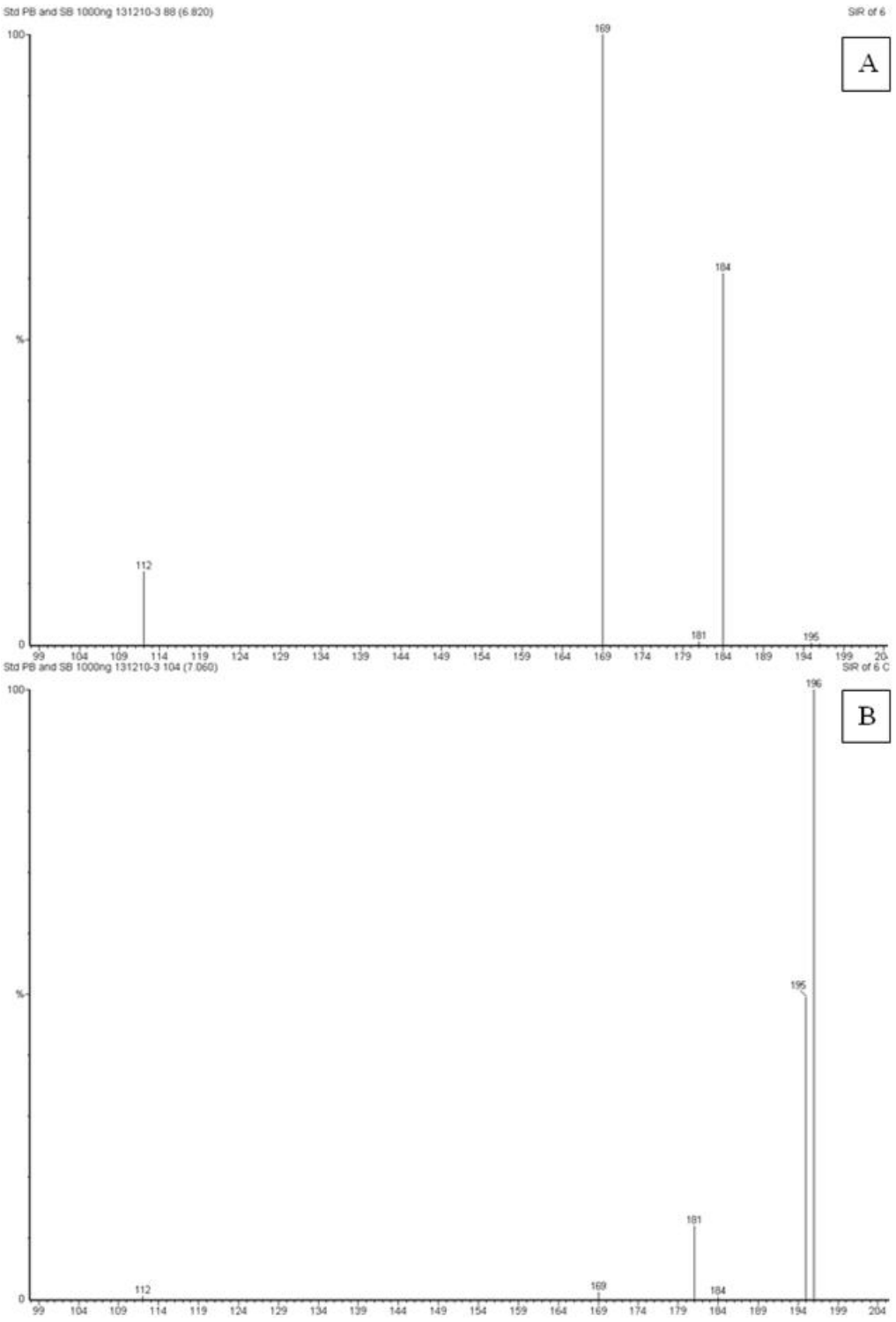


Figure 26: Mass spectra (SIM mode) of (A) PB and (B) SB

3.10 DCIT, CIT, NTRIP and AMI Analysis by UPLC-DAD

On the other hand, AMI, CIT and selected metabolites were analyzed by UPLC-DAD. Figure 27 shows a chromatogram containing the UV absorbance all four analytes (100 ng/mL) and the ISTD (250 ng/mL) at 240 nm, with retention times of approximately 2.04, 2.22, 3.97, 4.37 and 3.33 min for DCIT, CIT, NTRIP, AMI and DMI, respectively. Chromatograms of negative controls show minimum background at these retention times (figure 28). Analytes were identified by a combination of retention time (± 3 standard deviation in relation to standard samples) and their specific UV absorbance spectrum. Spectra of parent drug and metabolite can be very similar (figure 29), it is therefore very important to pay attention to the retention time when identifying peaks.

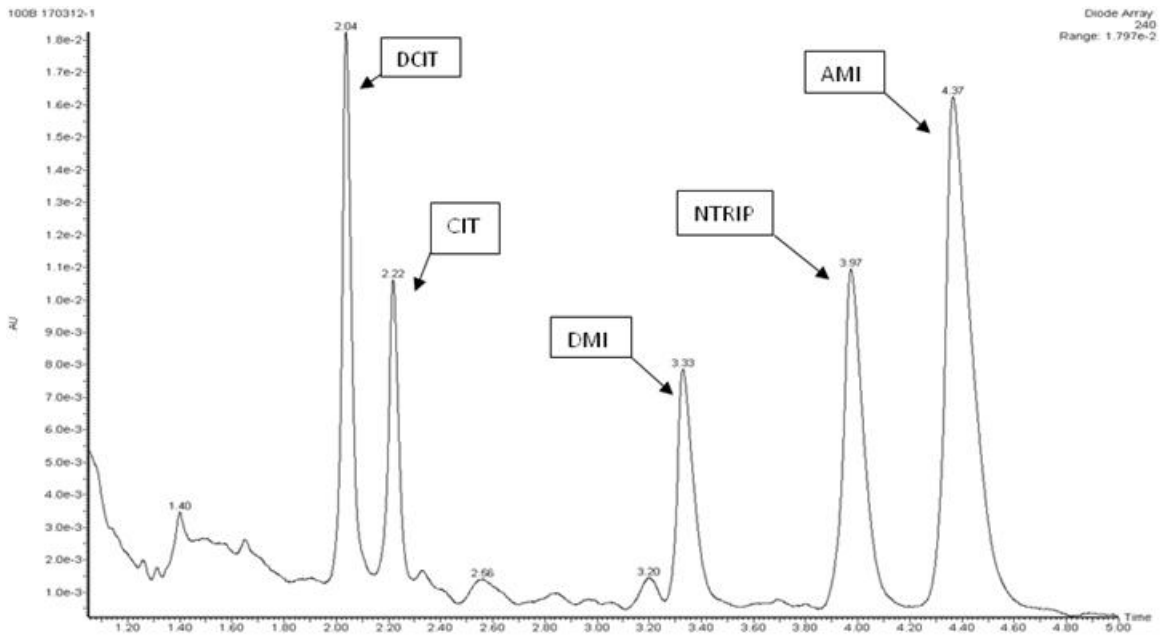


Figure 27: UPLC-DAD chromatogram of BTE spiked with 100 ng/mL of DCIT (2.04 min), CIT (2.22 min), NTRIP (3.97 min), AMI (4.37 min) and 250 ng of the internal standard DMI (3.33 min) at 240 nm

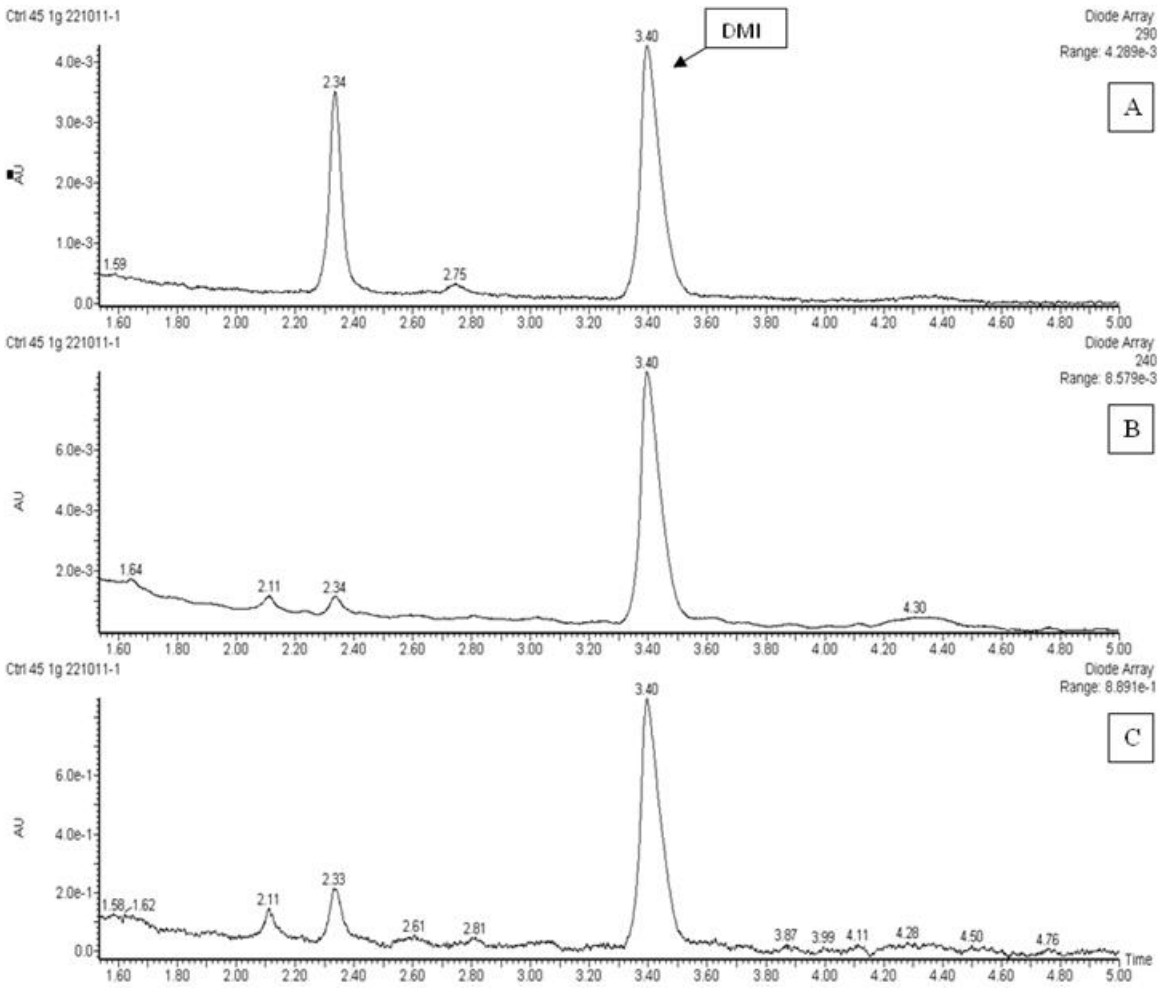
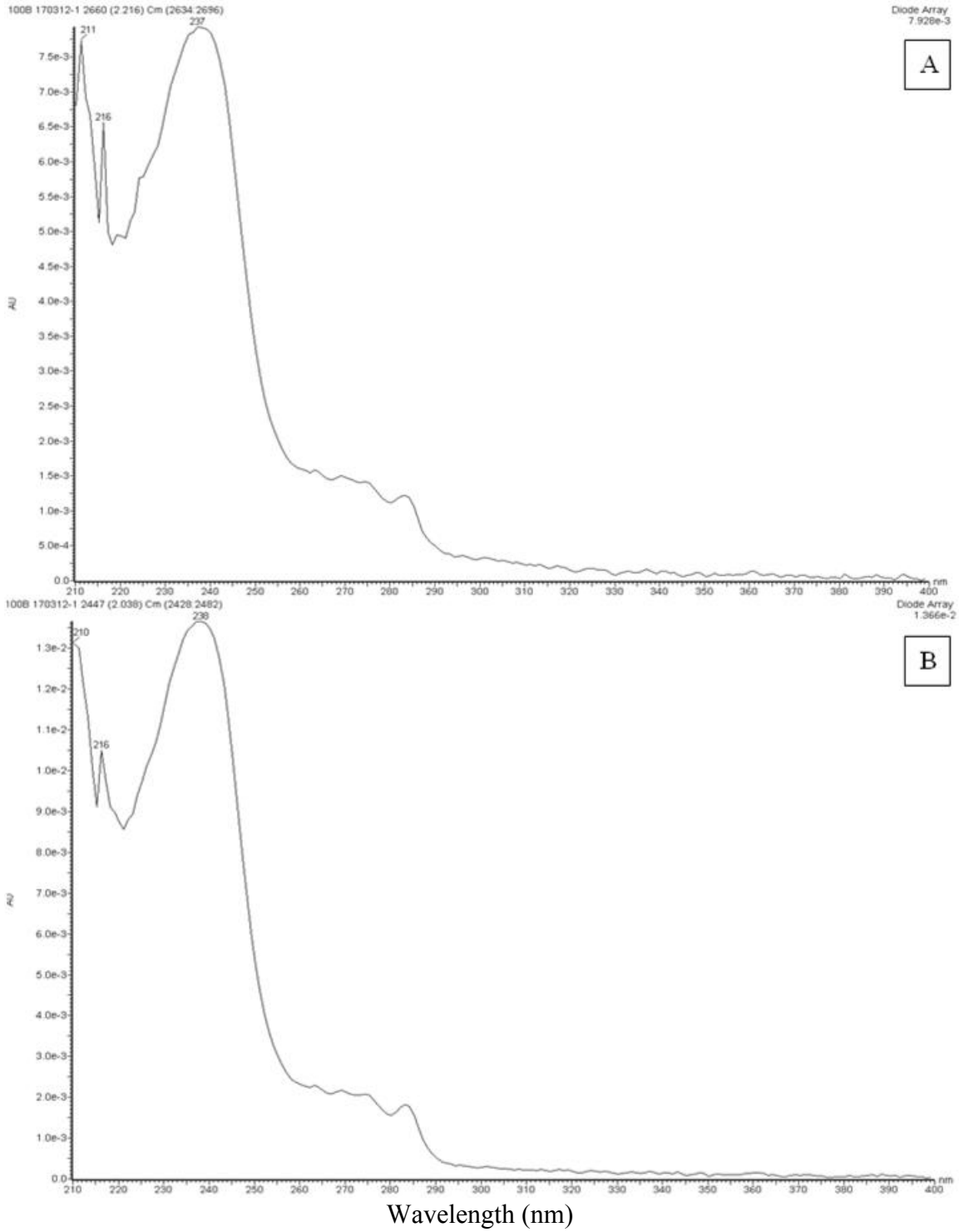


Figure 28: UPLC-DAD chromatogram of 1 g sample of control pig bone after 45 min of ultrasonic agitation (A) at 290 nm, (B) at 240 nm and (C) ranging from 210-400 nm



Wavelength (nm)

Figure 29: UV absorbance spectra of (A) CIT and (B) DCIT

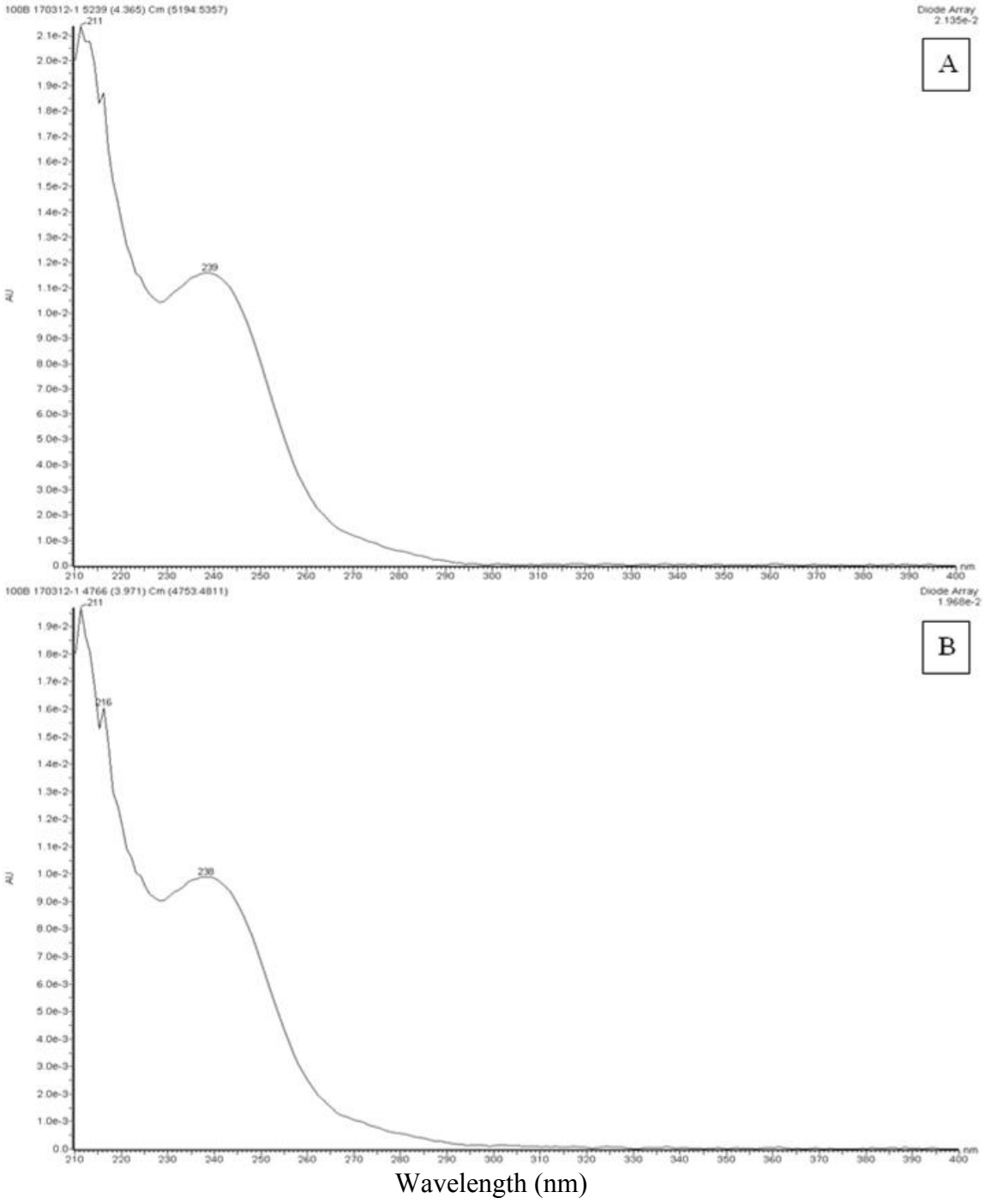


Figure 30: UV absorbance spectra of (A) AMI and (B) NTRIP

As seen in figure 31 B and C, an interference peak was eluted along with DMI in the positive pig samples. Obtaining the spectrum at 240 nm did not help (figure 31 B) since the spectrum of the

unknown compound also shows a strong absorbance at this wave length (figure 32 B). In order to overcome the interference, a different wave length was chosen for DMI; 290 nm (figure 32 A). When overlaying the two spectra, it is clear the interference is minimized when using 290 nm wavelength (figure 32 C).

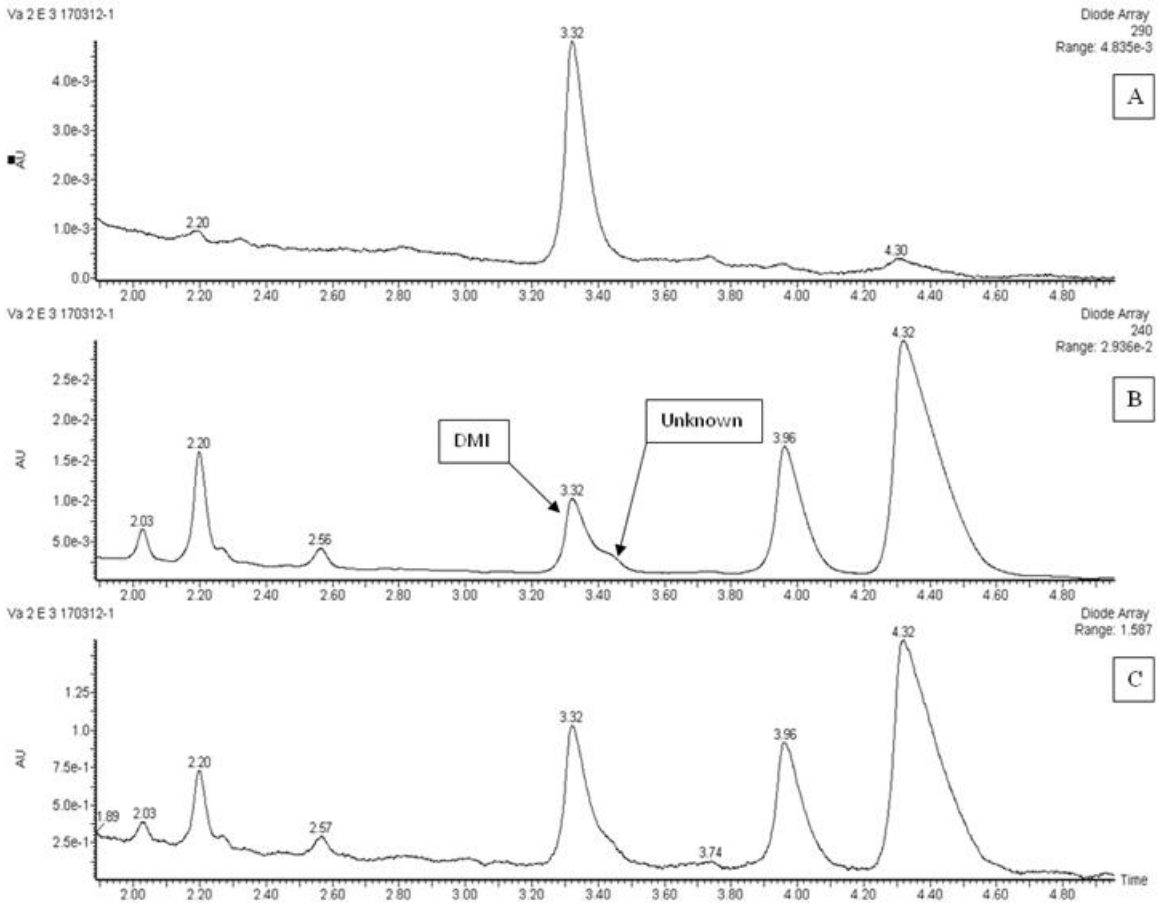


Figure 31: UPLC-DAD chromatogram of 2 g sample of pig bone after 3 min of microwave irradiation (A) at 290 nm, (B) at 240 nm) and (C) ranging from 210-400 nm

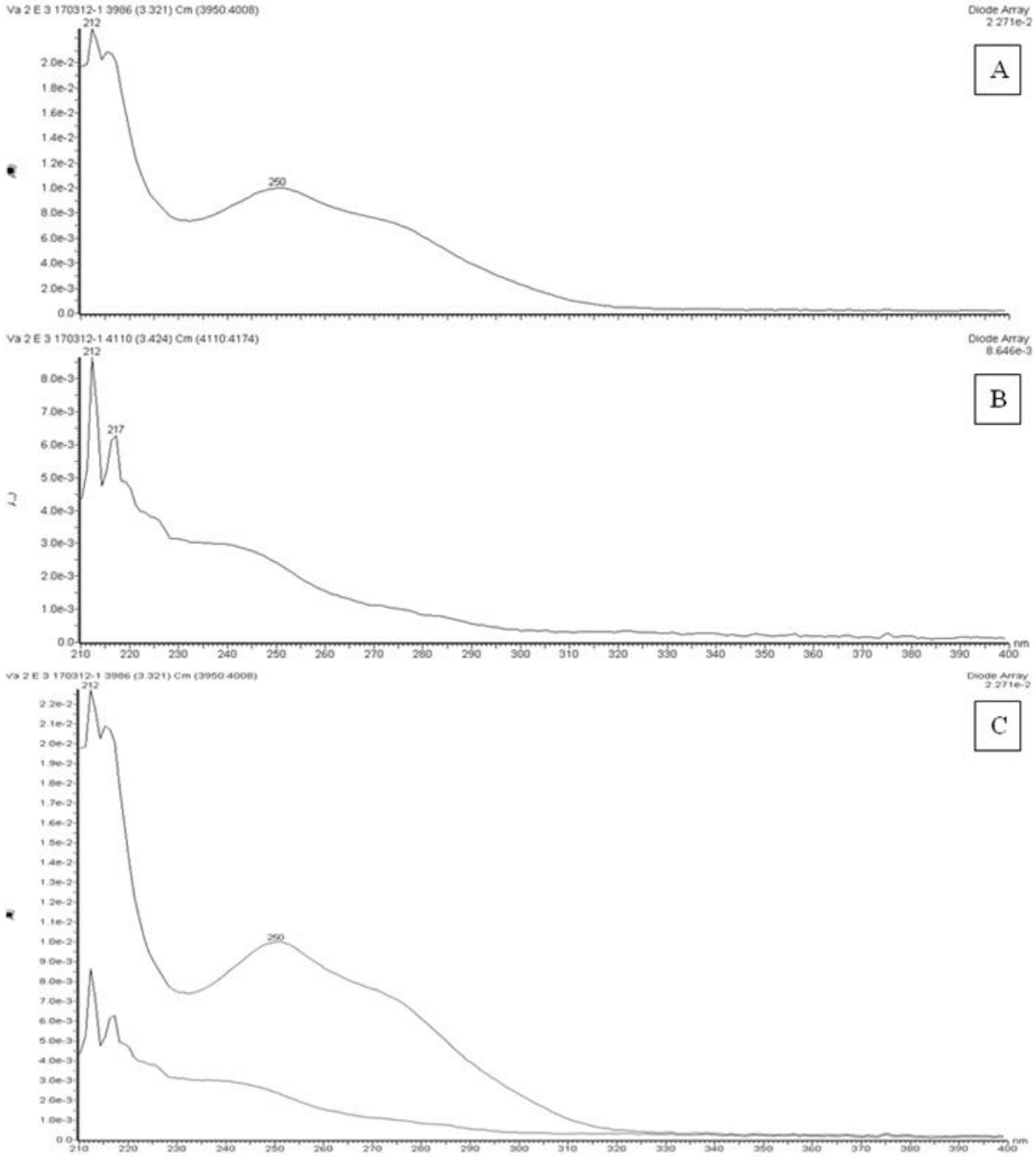


Figure 32: UV absorbance spectra of (A) DMI, (B) unknown compound and (C) overlay of both compounds

Chapter 4

4 Conclusion

4.1 Implication of the Research

The results show that methanol was a better extraction solvent for all three isolation methods (figure 17 A-D) and mass did not have an effect on the mass-normalized analyte yield. Certain analyte (DCIT and CIT) recovery levels were higher when extracted with less extraction solvent for USE, while more solvent was necessary for passive incubation, making USE a more cost effective and environmentally friendly technique (figure 22). Full microwave power resulted in higher recovery levels of some analytes (NTRIP and AMI) (figure 23), while samples subjected to Dg H_2O + Dg MeOH, resulted in higher yields on one occasion (AMI) (figure 24). When comparing maximum yield, a general trend indicated that MAE obtained higher analyte recovery compared to passive extraction and USE (figure 22).

Finally, a total of 30, 15 and 45 min were required to achieve maximum recovery by standard passive extraction, microwave irradiation and ultrasound agitation, respectively (figure 18-21), therefore making standard passive extraction and MAE more time effective. It is clearly demonstrated by these results that the extraction time required to isolate analytes of forensic toxicological interest by passive incubation from skeletal tissue is much shorter than what has been reported in the literature thus far.

4.2 Future Work

Drug analysis in skeletal tissue is still relatively new to forensic toxicology; therefore much more research is required. The comparison of samples submitted to standard passive extraction with manual vortexing and without vortexing should be considered to observe the influence of sample mixing in respect to analyte yield. The use of smaller sample mass (ex: 0.5 g) is also worth investigating.

It would be interesting to compare the analyte yield from samples irradiated in a domestic microwave to those submitted to laboratory-grade MAE. Also, the comparison of analyte recovery from closed- and open-vessel systems would be important to note.

In addition, it would also be interesting to examine the difference in analyte recovery from samples extracted by ultrasound agitation using different frequencies. Finally, the comparison of analyte extraction yield and rate from a water bath to a probe should also be investigated.

References

1 McGrath, K.K. and Jenkins, A.J. (2009) Detection of drugs of forensic importance

2 McIntyre, I.M., King, C.V., Boratto, M. et *al.* (2000) Postmortem drug analysis in bone and bone marrow. *Therapeut. Drug Monit.* **22(1):** 79-83.

3 Guillot, E., de Mazancourt, P., Durigon, M. *et al.* (2007) Morphine and 6-acetylmorphine concentraitons in blood, brain, spinal cord, bone marrow and bone after lethal acute or chronic diacetylmorphine administration to mice. *Forensic Sci. Inr.* **166:** 139-144.

4 Levine, B. (2006) Postmortem Forensic Toxicology. In Levine, B. (eds), Principles of Forensic Toxicology, 2nd ed. Chapter 1. AACC Press, Washington, DC, pp.3-13.

5 Raisz, L.G. (1999) Physiology and pathophysiology of bone remodeling. *Clin. Chem.* **45(8):** 1353-1358

6 Cengiz, S., Ulukan, O., Ates I. et *al.* (2006) Determination of morphine in post-mortem rabbit bone marrow and comparison with blood morphine concentration. *Forensic Sci. Int.* **156(2-3):** 91-94.

7 Marks, S.C. and Odgren, P.R. (2002) Structure and development of the skeleton. In Bilezikian, J.P., Raisz, L.G. and Rodan, G.A., (eds), Principles of bone biology, 1st edi, Chapter 1. Academic Press, San Diego, CA, pp.3-6.

8 Klein-Nulend, J. and Bonewald, L.F., (2008) Osteocytes. In Bilezikian, J.P., Raisz, L.G. and Martin T.J. (eds) Principles of bone biology, 3rd edi., Chapter 8. Academic Press, San Diego, CA, pp.153-174.

9 Martin, R.B., Burr, R.B. and Sharkey, N.A., (2007) Skeletal Biology. In Martin, R.B., Burr, R.B. and Sharkey, N.A. (eds), Skeletal Tissue Mechanics, 1st ed, Springer-Verlag Press, New York, NY, pp.29-41.

10 Winek, C.L., Westwood, S. and Wahba, W.W. (1990) Plasma versus bone marrow desipramine: A comparative study. *Forensic Sci. Int.* **48:** 49-57.

11 VandenBoer, T.C., Grummett, S.A. and Watterson, J.H. (2008) Utility of immunoassay in drug screening in skeletal tissue : Sampling considerations in detection of ketamine exposure in femoral bone and bone marrow following acute administration using ELISA. *J. Forensic Sci.* **53(6):** 1474-1482.

12 Watterson, J.H. and VandenBoer, T.C. (2008) Effects of tissue type and the dose- death interval on the detection of acute ketamine exposure in bone and marrow with solid-phase extraction and ELISA with LC/MS/MS confirmation. *J. Anal. Toxicol.* **32(8):** 631-638.

13 Watterson, J.H. and Botman, J.E. (2009) Detection of acute diazepam exposure in bone and marrow: Influence od tissue type and the dose-death interval on sensitivity of detection by ELISA with liquid chromatography tandem mass spectrometry confirmation. *J. Forensic. Sci.* **54(3):** 708-714.

14 Desrosiers, N.A., Betit, C.C. and Watterson, J.H. (2009) Microwave-assisted extraction in toxicological screening of skeletal tissues. *Forensic Sci. Int.* **188:** 23-30.

15 Terazawa, K. and Takatori, T. (1982) Determination of aminopyrine and cyclobarbital from a skeleton by radioimmunoassay. *J. Forensic Sci.* **27(4):** 844- 847.

16 Raikos, N., Tsoukali, H. and Njau, S.N. (2001) Determination of opiates in post-mortem bone and bone marrow. *Forensic Sci. Int.* **123:** 140-141.

17 Benko, A. (1985) Toxicological analysis of amobarbital and glutethimide from bone tissue. *J. Forensic Sci.* **30:** 708-714.

18 Wohlenberg, N., Lindsay, T., Backer, R et *al.* (1992) Case notes from members: Nortriptyline in maggots, muscle, hair, skin and bone in skeletonised remains. *TIAFT Bull.* **22:** 19-22.

19 Horak, E.L., Felo, J.A and Jenkins, A.J. (2002) Postmortem tissue distribution of olanzapine and citalopram in an overdose. *American Academy of Forensic Sciences* 54th Annual Meeting. Atlanta, GA.

20 Horak, E.L. and Jenkins, A.J. (2005) Postmortem tissue distribution of olanzapine and citalopram in a drug intoxication (Case Report). *J. Forensic Sci.* **53(3):** 679-681.

21 McGrath, K.K. and Jenkins, A.J. (2004) Analysis of post-mortem bone/bone marrow specimens for drug of importance in forensic toxicology. *J. Anal. Toxicol.* **29(5):** 503.

22 Watterson, J.H., Donohue, J.P. and Betit, C.C. (2012) Comparison of relative distribution of ketamine and norketamine in decomposed skeletal tissues following single and repeated exposure. *J. Anal. Toxicol.* **36(6):** 429-433.

23 Pellegrini, M., Casa A., Marchei, E. et *al.* (2005) Development and validation of a gas chromatography-mass spectrometry assay for opiates in human teeth. *J. Pharmacol. Biomed. Anal.* **40(3):** 662-668.

24 Cattaneo, C., Gigli F., Lodi, F. et *al.* (2003) Detection of morphine and codeine in human teeth: An aid in the identification and study of human skeleton remains. *J Forensic Odonto-stomatol.* **21(1):** 1-5.

25 Neas, E.D. and Collins, M.J., (1988) Microwave Heating – Theorical concepts and equipment design. In Kingston H.M. and Jassie L.B (eds), Introduction to microwave sample preparation – Theory and practice, 1st edi, Chapter 2. American Chemical Society Professional Reference Book, Washington, DC, pp.7-32.

26 Lidström, P., Tiernay, J., Wathey, B. et *al.* (2001) Microwave assisted organic synthesis – A review. *Tet*. **57:** 9225-9283.

27 Hesek, J.A. and Wilson, R.C. (1974) Use of a microwave in in-process control. *Anal. Chem.* **46(8):** 1160.

28 Abu-Samra, A., Morris, S. and Koirtyohann, S.R. (1975) Wet ashing of some biological samples in a microwave oven. *Anal. Chem.* **47(8):** 1475-1477.

29 Nadkirni, R.A. (1984) Applications of microwave oven sample dissolution in analysis. *Anal. Chem.* **56:** 2233-2237.

30 Eskilsson, C.S. and Björklund, E. (2000) Analytical-scale microwave-assisted extraction. *J. Chrom. A.* **902:** 227-250.

31 Geyde, R., Smith, F. and Westway, K. (1986) The use of microwave ovens for rapid organic synthesis. *Tet. Lett.* **27(3):** 279-282.

32 Jin, Q., Liang, F., Zhang, H. et *al.* (1999) Application of microwave techniques in analytical chemistry. *Trends Anal. Chem.* **18(7):** 479-484.

33 Morales-Rubio, A., Julian-Ortiz, J.V., Salvador, A. et *al*. (1994) Hydrolysis of benzodiazepines in a microwave oven and ultraviolet derivative analysis of their benzophenones. *Microchem. J.* **49:** 12-19.

34 Lopez-Avila, V. and Young, R. (1994) Microwave assisted extraction of organic compounds from standard reference soils and sediments. *Anal. Chem.* **66(7):** 1097-1106.

35 Thompson, S., Budzinski,H., LeMenach, K. et *al.* (2002) Multi-residue analysis of polycyclic aromatic hydrocarbons, polychlorobiphenyls, and organochlorine pesticides in marine sediments. *Anal. Bioanal. Chem.* **372:** 196-204.

36 Lopez-Avila, V. and Benedicto, J. (1996) Microwave-assisted extraction combined with gas chromatography and enzyme-linked immunosorbent assay. *Trends Anal. Chem.* **75(8):** 334-341.

37 Mandal, V., Mohan, Y. and Hemalatha, S. (2007) Review Article: Microwave Assisted Extraction – An Innovative and Promising Extraction Tool for Medicinal Plant Research. *Pharmacognosy Reviews*. **1(1):** 7-18.

38 Chen, S.S. and Spiro, M. (1994) Study of microwave extraction of essential oil constituents from plant materials. *J. Microwave Power.* **29(4):** 231-241.

39 Madej, K. (2009) Microwave-assisted and cloud-point extraction in determination of drugs and other bioactive compounds. *Trends Anal. Chem.* **28 (4):** 436-446.

40 Letellier, M. and Budzinski, H. (1999) Microwave assisted extraction of organic compounds. *Analusis.* **27:** 259-271.

41 Franke, M., Winek, C.L. and Kingston, H.M. (1996) Extraction of selected drug from serum using microwave irradiation. *Forensic Sci. Int.* **81:** 51-59.

42 Jassie, L., Revesz, R., Kierstead T. et *al.* (1997) Microwave-Assisted Solvent Extraction. In Kingston, H.M. and Haswell, S. (eds), Microwave Enhanced Chemistry: Fundamentals, sample preparation and application, 1st edi, Chapter 12. American Chemical Society Professional Reference Book, Washington, DC, pp. 569-609.

43 Verma, S.C., Nigam, S., Jain, C.L. et *al.* (2011) Microwave-assisted extraction of gallic acid in leaves of *Eucalyptus x hybrida* Maiden and its quantitative determination by HPTLC. *Der Chemica Sinica.* **20(2):** 268-277.

44 Baghurst, D.R. and Mingos, D.M.P. (1992) Superheating Effects Associated with Microwave Dielectric Heating. *J. Chem. Soc. Chem. Commun.* **1992(9):** 674-677.

45 Luque-Rodriguez, J.M., Luque de Castro, M.D. and Perez-Juan, P. (2005) Extraction of Fatty Acids from Grape Seed by Superheated Heaxane. *Talanta.* **68(1):** 126-130.

46 Fernández, P., Lago, M., Lorenzo, R.A. et *al.* (2009) Optimization of a rapid microwave-assisted extraction method for the simultaneous determination of opiates, cocaine and their metabolites. *J. Chrom. B.* **877:** 1743-1750.

47 Watterson, J.H. and Desrosiers, N.A.(2011) Examination of the effect of dose-death interval on detection of meperidine exposure in decomposed skeletal tissues using microwave-assisted extraction. *Forensic. Sci. Int.* **207:** 40-45.

48 Suslick, K.S. (1989) The chemical effect of ultrasound. *Sci. Am.* **260(2):** 80-86.

49 Luque-García, J.L. and Luque de Castro, M.D. (2003) Ultrasound: a powerful tool for leaching. *Trends Anal. Chem.* **22(1):** 41-47.

50 Miller-Ihli, N.J. (1988) Slurry sample preparation for simultaneous multi-element furnace atomic absorption spectrometry. *J. Anal. At. Spectrom.* **3(1):** 1414-1419.

51 Santos, D.J., Krug, F.S., Pereira, M.G. et *al.* (2006) Currents on ultrasound-assisted extraction for sample preparation and spectroscopic analyte determination. *Appl. Spectros. Rev.* **41(3):** 305-321.

52 Wibetoe, G., Takuwa, D.T., Lund, W. et *al.* (1999) Coulter particle analysis used for studying the effect of sample treatment in slurry sampling electrothermal atomic absorption spectrometry. *Fresenius J. Anal. Chem.* **363(1):** 46-54.

53 Vinodgopal, K., Peller, J., Makogon, O. et *al.* (1998) Ultrasonic mineralization of a reactive textile azo dye, Remazol Black B. *Wat. Res.* **32(12):** 3646-3650.

54 Buldini, P.L., Mevoli, A. and Sharma, J.L. (1998) LA-ICP-MS, IC and DPASV-DPCSV determination of metallic impurities in solar-grade silicon. *Talanta*. **47:** 203-212.

55 Fransson, B. and Ragnarsson, U. (1998) Separation of enantiomers of α-hydroxyl acids by reversed-phase liquid chromatography after derivatization with 1-(9-fluorenyl)ethylchlorofomate. *J. Chrom. A.* **827:** 31-36.

56 Boudjouk, P. (1986) Synthesis with ultrasonic waves. *J. Chem. Educ.* **63(5):** 427-429.

57 Chemat, F., Huma, Z. and Khan, M.K. (2011) Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultras. Sonochem.* **18:** 813-835.

58 Capelo, J.L., Maduro, C. and Vilhena, C. (2005) Discussion of parameters associated with the ultrasonic solid-liquid extraction for elemental analysis (total content) by electrothermal atomic absorption spectrometry: An overview. *Ultras. Sonochem.* **12:** 225-232.

59 Carvalha, L.R.F., Souza, S.R., Martinis, B.S. et *al*. (1995) Monitoring of the ultrasonic irradiation effect on the extraction of airborn particulate matter by ion chromatography. *Anal. Chim. Acta.* **317:** 171-179.

60 Shirsath, S.R., Sonawane, S.H. and Gogate, P.R. (2012) Intensification of extraction of natural products using ultrasonic irradiation – A review of current status. *Chem. Eng. Processing.* **53:** 10-23.

61 Babic, S., Petrovic, M., Kaštelan-Macan, M. (1998) Ultrasonic solvent extraction of pesticides from soil. *J. Chrom. A.* **823:** 3-9.

62 Tian, Y., Xu, Z., Zheng, B. et *al.* (2013) Optimization of ultrasonic-assisted extraction of pomegranate (Punica granatum L.) seed oil. *Ultras. Sonochem.* **20:** 202-208.

63 Tor, A., Aydin, M.E. and Özcan, S. (2006) Ultrasonic solvent extraction of organochlorine pesticides from soil. *Anal. Chim. Acta.* **559:** 173-180.

64 Rothe, M. and Pragst, F. (1995) Solvent optimization for the direct extraction of opiates from hair samples. *J. Anal. Toxicol.***19:** 236-240.

65 Gorcszynski, L.Y. and Melbye, F.J. (2001) Detection of benzodiazepines in different tissues, including bone, using a quantitative ELISA assay. *J. Forensic Sci.* **46(4):** 916-918.

66 Rana, S., Uralets, V.P. and Ross, W. (2008) A new method for simultaneous determination of cyclic antidepressants and their metabolites in urine using enzymatic hydrolysis and fast gas chromatography-mass spectrometry. *J. Anal. Toxicol.* **32:** 355-363.

67 Baeck, S.K., Lira, M.A, Park, S.Y, *et al.* (2000) Blood concentrations of amitriptyline and its metabolite in rats after acute oral administration of amitriptyline. *J. Anal. Toxicol.* **24:** 271-274.

68 Melanson, S.E.F., Leetewandroski, E., Griggs, D.A. *et al.* (2007) Interpreting tricyclic antidepressant measurements in urine in an emergency department setting : comparison of two qualitative point-of-care urine tricyclic antidepressant drug immunoassays with quantitactive serum chromatographic analysis. *J. Anal. Toxicol.* **31:** 270-275.

69 Martinez, M.A., Sanchez de la Torre, C. and Almarza, E. (2003) A comparative SPE study for the simultaneous determination of fluoxetine, amitriptyline, trimipramine, maprotiline, clomipramine and trazodone in whole blood by capillary gas-liquid chromatography with nitrogen-phosphorus detector. *J. Anal. Toxicol.* **27:** 353-358.

70 Breyer-Pfaff, U., Gaertner, H.J., Kreuter, F. et *al.* (1982) Antidepressive effect and Zsbvcxz pharmacokinetic of amitriptyline with consideration of unbound drug and 10-hydroxynortriptyline plasma levels. *Psypharmacol.* **76:** 240-244.

71 Coudore, F., Besson, A., Eschalier, A. et *al.* (1996) Plasma and brain pharmacokinetic of amitriptyline and its demethylated and hydroxylated metabolites after one and six half-life repeated administrations to rats. *Gen. Pharmacol*. **27(2):** 215-219.

72 Lee, X.-P., Hasegawa, C., Kumazawa, T. et *al.* (2008) Determination of Tricyclic antidepressants in human plasma using pipette tip solid-phase extraction and gas chromatography-mass spectrometry. *J. Sep. Sci.* **31:** 2265-2271.

73 Goldman, R.H., Stason, W.B., Park, S.Y., *et al.* (2010) Low-dose amitriptyline for

treatment of persistent are pain due to repetitive use. *Pain.* **149:** 117-123.

74 Baselt, R.C. and Cravey, R.H. (2004) Disposition of Toxic Drugs and Chemicals in Man. 4th edi. Biomedical Publications Press. Foster City, CA.

75 Dorian, P., Sellers, E.M., Reed, K.L. et *al.* (1983) Amitriptyline and ethanol : Pharmacokinetic and pharmacodynamic onteraction. *Eur. J. Clin. Pharmacol.* **25:** 325-331.

76 Bynum, N.D., Poklis, J.L., Gaffney-Kraft, M. et *al.* (2005) Postmortem distribution of tramadol, amitriptyline and their metabolites in a suicidal overdose. *J. Anal. Toxicol.* **29:** 401-406.

77 Lewis, R.J., Angier, M.K., Johnson, R.D. et *al.* (2011) Analysis of citalopram and desmethylcitalopram in postmortem fluids and tissues using liquid chromatography-mass spectrometry. Civil Aerospace Medical Institute Federal Aviation Administration. Oklahoma City, OK. *www.faa.gov/data_research/ research/med_humanfacs/oamtechreports/2010s/media/201117.pdf*

78 Jenkins, A.J. and Gubanich, K. (2002) Diposition of citalopram in biological specimens from post-mortem cases. *J. Forensic Sci.* **47(1):** 159-164.

79 Kugelberg, F.K., Kingback, M., Carlsson, B. et *al.* (2005) Early-phase post-mortem redistribution of the enantiomers of citalopram and its demethylated metabolites in rats. *J. Anal. Toxicol.* **29:** 223-228.

80 Shapiro, H.M. (1985) Barbiturates in brain ischaemia. *British J. Anaesth.* **57(1):** 82-95.

81 Johnson, L.L. and Grag, U. (2010) Quantitation of Amobarbital, butalbital, pentobarbital, phenobarbital and secobarbital in urine, serum and plasma using gas chromatography-mass spectrometry (GC-MS). *Meth. Molecular Biol.* **603:** 65-74.

82 Higashi, H. and Nishi, S. (1982) Effect of barbiturates on the GABA receptor of cat primary afferent neurones. *J. Physiol.* **332:** 299-314.

83 Christensen, H.D., Barnett, L. and Carroll, F.I. (1973) Biological activity of pentobarbital metabolites. *J. Pharm. Sci.* **62(10):** 1722-1723.

84 Tang, B.K., Inaba, T. and Kalow W. N-hydroxylation of pentobarbital in man. (1977) *Drug Metab. Disp.* **5(1):** 71-74.

85 Baldeo, W.C., Gilbert, J.N. and Powell, J.W (1980). Multidose studies in the human metabolism of pentobarbital. *Eur. J. Drug Metab. Pharmacokin.* **5(2):** 75-80.

86 Smith, R.B., Dittert, L.W., Griffen, W.O. et *al.* (1973) Pharmacokinetics of pentobarbital after intravenous and oral administration. *J. Pharmacokin. Biopharm.* **1(1):** 5-16.

87 Franke, J.P. and Zeeuw, R.A. (1998) Extraction procedures in systematic toxicological analysis. *J. Chrom. B.* **713:** 51-59.

88 Liu, R.H., McKeehan, A.M., Edwards, C. et *al.* (1994) Improved gas chromatography-mass spectrometry analysis of barbiturates in urine centrifuge-based solid-phase extraction, methylation, with d5-pentobarbital as internal standard. *J. Forensic Sci.* **39:** 1504-1514.

89 Martinez, M.A, Sanchez de la Torre, C. and Almarza E. (2004) A comparative SPE study for the simultaneous determination of fluvoxamine, mianserin, doxepin, citalopram, paroxetine, and etoperidone in whole blood by capillary gas-liquid chromatography with nitrogen-phosphorus detector. *J. Anal. Toxicol.* **28(3):** 174-180.

90 Winek, C.L., Costantino, G., Wahba, W.W. et *al.* (1985) Blood versus bone marrow pentobarbital concentrations. *Forensic Sci. Int.* **27:** 15-24.

91 Ulrich, S., Isensee, T. and Peter, T. (1996) Simultaneous determination of amitriptyline, nortriptyline and four hydroxylated metabolites in serum by capillary gas-liquid chromatography with nitrogen-phosphorus selective detection. *J. Chrom. B: Biomed. Appl.* **685(1):** 81-89.

92 Hedges, R.E. (2002) Bone diagenesis: An overview of processes. *Archeometry*. **44(3):** 319-328.

93 Watterson, J.H., Desrosiers, N.A., Betit, C.C., et *al.* (2010) Relative distribution of drugs in decomposed skeleton tissue. *J. Anal. Toxicol.* **34(8):** 510-515.

94 Nagata, T., Kimura, K., Hara, K. et *al*. (1990) Methamphetamine and amphetamine concentrations in postmortem rabbit tissues. *Forensic Sci. Int.* **48:** 39-47.

95 Wyman, J.F., Dean, D.E., Yinger, R. *et al.* (2011) The temporal fate of drugs in decomposing porcine tissue. *J. Forensic Sci.* **56(3):** 694-699.

96 Watterson, J.H., Snow, M.S. (2008) Detection of Acute Pentobarbital Exposure in Tibial Bone and Marrow by Solid-phase Extraction, ELISA and Positive Ion Chemical Ionization Gas Chromatography–Mass Spectrometry. *Society of Forensic Toxicologists* Conference, Phoenix, AZ. (Poster P52).